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13. ABSTRACT *(Maximum 200 Words)*

Soldiers face a broad range of occupational/environmental threats which are hazardous to central nervous system (CNS) function. One of these, exposure to prolonged, extreme stress, has been recently associated with neuronal loss. Vietnam veterans with PTSD have decreased hippocampal volume on MRI, as well as decreased scores on standardized tests of memory. Short term (recent) memory function has been localized to the hippocampus. Humans exposed to extreme stress for sustained periods have suffered deterioration of memory and inability to concentrate, as well as CNS atrophy. Chronic stress in several species, including mouse, rat, tree shrew and monkey, have been reported to develop alterations in hippocampal morphology, including apical dendritic atrophy, depletion of vesicles in mossy fiber terminals, as well as actual loss of neurons. A number of stress-induced molecular pathophysiological mechanisms leading to neuronal loss/dysfunction have been described, including: (1) excessive release of excitatory amino acids (in particular, glutamate), leading to massive influx of extracellular calcium into neurons; (2) excessive release of adrenal glucocorticoids, which exacerbates the effect of excitatory amino acids. We have successfully characterized acute social defeat in the mouse as a model for eliciting high plasma levels of adrenal glucocorticoids, inducing high levels of stress and persistent avoidance behavior, as well as increased brain regional levels of mRNA for corticotrophin-releasing hormone and decreased hippocampal levels of mRNA for brain-derived neurotropic factor BDNF. We will characterize the hippocampal neuronal changes induced in this paradigm, and then after chronic stress attempt to block them with a range of pharmaceutical interventions, including neurosteroids, antagonists to CRH and antagonists to excitatory amino acids.

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INTRODUCTION

There is now considerable data showing that severe stress can cause lasting pathology, including objective evidence of neuronal atrophy and/or loss (Magarinos and McEwen 1995a, 1995b; Magarinos et al. 1996Uno et al., 1989; Mizoguchi et al., 1992). In both human and animal studies, these abnormalities have been accompanied by measurable deficits in memory (Bremner et al. 1995; Luine et al. 1994, 1996). Most published work has been accomplished using restraint stress in the rat (Conrad et al., 1996; Luine et al. 1994, 1996). We proposed to examine stress-induced memory deficits and associated hippocampal neuroanatomical changes in the very robust social defeat (SD) model and to examine a number of novel pharmacological interventions as neuroprotective measures. Defeat in mice is a severe naturalistic stressor, and may be especially relevant to the stress of combat, PTSD and stress-induced changes in hippocampal (HC) morphology. SD induces persistent behavioral changes. Ones session of three 2 min bouts in our acute SD paradigm is followed by a suppression of normal territorial marking that persists for three weeks (Lumley et al., 1999). The proposed research has four major aims. We will employ repeated SD to determine the parameters required to induce (1) maximal deficits in Y maze spatial memory performance and (2) morphological changes in HC. In addition, we will (3) compare the effects of SD to those of repeated restraint on memory and HC morphology. Finally we will (4) examine the efficacy of a number of pharmaceutical interventions in preventing exaggerated fear responses, memory deficits and neurochemical and morphological changes in the HC induced via the acute and chronic SD paradigm.

BODY OF WORK

The principle objectives in year one were to determine: the parameters of social defeat (SD) that induce maximal impairment of memory; the time course of memory impairment; whether Y-maze testing affects behavior in the modified resident-intruder test; the validity of repeated Y-maze testing in the SD mouse; the effects of treatment with pharmacological (e.g. anxiolytic) agents on acute SD.

In brief, we discovered (1) a major flaw in the Y-maze equipment as designed and manufactured by Coulbourn Inst., which we corrected; (2) a major problem with the initial animal supplier (Charles River), corrected by changing suppliers; (3) that repeated daily Y-maze testing is problematic and that intermittent testing will be required; (4) anxiolytic effects of two pharmaceutical agents (one novel compound and one well-known anxiolytic) in acute SD; (5) strain differences in response to anxiolytics; (6) that dehydration may have been an unsuspected confound in experiments previously published by others. A few obstacles in meeting these goals are indicated in section V. The results of our first year experiments were met as follows:

I. We will determine whether repeated testing in the Y-maze is valid in socially stressed mice. We planned to use the Y-maze test of short-term memory 24 hours after completion of each series of stress exposures.

The Y-maze test is based on spontaneous exporatory behavior and the finding that rodents, including mice, spend more time in a novel environment than in a familiar environment. On trial one, mice have access to the start arm and to the "familiar arm" of the three-arm maze. On trial two, mice have access to all three arms, typically spending more time in, with the shortest latency to enter, the novel arm. We tested DBA/2 mice in the Y-maze for 21 consecutive days. Half of the mice received SD each afternoon and were tested the following morning in the Y-maze, and half of the mice were not defeated (NOSD). Data were analyzed by a repeated measures analysis with defeat status as a between factor. Although there were no significant differences between SD and NOSD mice, SD mice tended to be less active than NOSD mice within a few days of successive defeat. However, with repeated tests, NOSD mice also became less active in this test, possibly habituating to daily testing. Habituation by NOSD mice may have masked the effects of SD on exploratory activity (see Figure 1). Although others have used the Y-maze test following chronic restraint stress (Conrad et al., 1996) and chronic corticosterone administration (Bardgett et al., 1994) without affecting exploratory activity, stress-induced immobility or behavioral inhibition may be a potential confound in measuring memory in the Y-maze test. Rats that received acute social defeat displayed decreased locomotor activity in a novel environment, an open field test (Meerlo et al., 1997a) and in a running wheel (Meerlo et al., 1997b), days following social defeat. Whether chronic social defeat induces adaptation or impairs exploratory activity is unclear. Since stress-induced immobility is of concern, we plan to add the Morris water maze as an additional behavioral test to determine the time course of chronic social stress on memory impairment and morphological changes. In the forced swim test, we have observed that acute SD in mice induces transient immobility (Hebert et al., 1998). Twenty-four hours post-SD, there were no effects of defeat on immobility. Therefore, stress-induced immobility may be less confounding in the Morris water maze test, which also requires swim behavior. The Morris water maze has been used extensively and is considered a useful test of spatial memory in mice (i.e. Tecott et al., 1998; Guillou et al., 1999). Rousse et al. (1997) showed that transgenic mice with impaired glucocorticoid receptor function performed poorly in the Morris water maze, further supporting a modulatory role of glucocorticoids on spatial memory. Stress-induced glucocorticoids contribute to hippocampal dysfunction and impairment of spatial memory (reviewed in McEwen, 1999). Integrity of the hippocampus is considered necessary for spatial memory (Aggleton et al., 1986; Jarrard, 1993), including performance in the Morris water maze (Morris et al., 1982).

There may be a turning preference in mice. Each day, the "novel or unfamiliar" arm was alternated with each day of testing, such that the novel arm from the preceding day was designated the familiar arm. The mazes were also rotated and the visual cues were altered. However, some DBA/2 mice tended to spend more time in one particular arm regardless of whether it was the novel arm or the familiar arm. Figure 2 illustrates how both SD and NOSD mice tended to alternate daily their time spent in the novel relative to familiar arm. This may indicate that the mice have a preference for turning to one side (ie. spending most of the time in the arm to the right of the start). Alternatively, there may have been carryover effects from performance on the preceding day. There was much variability among test days in all DBA/2 subjects (see Figure 3). We concluded that daily testing of DBA/2 mice in the Y-maze test along with daily defeats was not valid.

We tested whether there were strain differences in behavior in the Y maze in order to determine whether one strain would be preferable for use in the social stress studies. For the baseline test, a t-test was performed in all mice between duration spent in the familiar versus the novel arm; there was a non-significant tendency for mice to spend a greater percentage of time in the novel arm (see Figure 4), as has been observed by others (Conrad et al., 1996). For strain differences, data were analyzed using t-tests between C57BL/6 and DBA/2 mice for each parameter. There was a non-significant trend for C57BL/6 mice to spend less time in the familiar arm on trial two, than did DBA/2 mice (see Figure 5). In addition, DBA/2 mice were significantly more active, as indicated by a greater number of total arm entries than had C57BL/6 mice (see Figure 6). Others (Crawley et al., 1997) have suggested that C57BL/6 mice may be more useful as subjects since they tended to have lower variability in behavior than did DBA/2 mice. Our findings of high activity in DBA/2 mice support this suggestion.

II. We will determine the time course of development of memory impairment following repeated social defeats. We hypothesized that memory impairment would predict the development of alterations in hippocampal morphology.

Since daily testing in the Y-maze resulted in habituation (see Figure 1), we tested mice in the Y-maze at the end of repeated stress exposures rather than daily. Rats that received 6 h restraint stress for 21 successive days displayed alterations in spatial memory (Conrad et al., 1996) and in hippocampal morphology (Magarinos and McEwen, 1995). Therefore, we exposed C57BL/6 mice to 21 days of social stress, followed by the Y-maze test and the resident-intruder test. These mice were from Charles River (see section V b) but were asymptomatic throughout these tests. Of particular interest, rats that received 21 days of 6 h restraint stress were food and water deprived during the stress period (Magarinos and McEwen, 1995). We were concerned that those rats may have been dehydrated. Dehydration is of high military relevance since dehydration can rapidly cause battle fatigue (U.S. Army Field Manual, 1994; Jones et al., 1995); S.L.A. Marshall is quoted as saying "No one ever told me that dehydration causes cowardice in its most abject form". In addition, dehydration tends to increase serum osmolarity, which induces release of vasopressin (AVP; reviewed in Jezova et al 1995). There are numerous reports that AVP affects both consolidation and retrieval of memory (reviewed in Bohus et al., 1993). The effects of AVP on memory vary depending on the time of administration (or endogenous release). In Vietnam Veterans with PTSD, intranasal AVP facilitated memory (Pitman et al., 1993). Similarly in mice, AVP administered immediately after SD increased submissiveness (Roche and Leshner 1979; Siegfried et al., 1984) and in rats, an AVP antagonist inhibited passive avoidance (Greidanus and DeWied, 1976); these authors suggested that AVP enhances memory for a stressful experience. However, when AVP was administered to mice prior to SD, mice displayed less submissive behavior on a post-test, suggesting AVP inhibited memory.

In the current experiment, we exposed mice to 21 days of 6 h of social stress, including food and water deprivation in order to be consistent with the McEwen restraint stress studies. However, to prevent the subject from being injured by the aggressor, we placed the subject mice within an enclosure in the aggressor's home cage and randomly exposed the subjects to brief episodes (1-5 bites, 0-3 times/day) of direct contact with the aggressor. This resulted in the subject being food and water deprived and psychologically stressed for 6 h, but not wounded. Compared to the severity of the social stress in our acute defeat paradigm (three two min. bouts, each within the home cage of a different attacker), we consider this a moderate stressor. Two groups of control mice were also tested. One group was food and water deprived for 6 h, but was not socially stressed. The other group was not food or water deprived, or socially stressed. Animals were returned to their home cages with food and water available *ad libidum* after each 6 h stress or deprivation session. After the 21st day of the stress exposure, the mice were provided a full 24 h home cage rest period with food and water available *ad libidum*. Data were analyzed for each parameter of the Y-maze and for behaviors in the resident-intruder test by a one way analysis of variance (ANOVA), with stress exposure as the between factor. Body weights were taken at the beginning of the stress experience (or water deprivation) and at the end of the experience. A repeated measures ANOVA was performed on the daily change in body weight, with stress exposure as a between factor. Mice that received social stress (and food and water deprivation) lost significantly more weight (10% of their normal body weight) throughout the stress period than non-deprived mice lost throughout this same time period (see Figure 7A). In addition, mice that were food and water deprived (no social stress) lost more weight during the 6 h deprivation period than control mice, but not as much as stressed mice. This weight loss was transient, since when weighed on the following day immediately prior to the experimental session, the animals had recovered their body weight. Control mice tended to gain weight (about ½ gram) during the 6 h test period. Surprisingly, the non-socially stressed dehydrated mice had increased activity in the Y-maze test, as demonstrated by significantly more entries into the novel arm (see Figure 7B), and a tendency for more entries into the familiar arm. The effects of dehydration on activity may have been mediated in part by increased AVP, which activates the autonomic nervous system and increases arousal (Koob et al 1985). Since the psychologically stressed group of mice was also dehydrated, yet did not display increased activity, other neurochemical changes, such as stress induced intra-amygdalar release of endogenouse corticotropin-releasing hormone (CRH) may have countered the effects of AVP. In rats, CRH administered intracerebroventricularly or into the amygdala induced freezing (Koob et al., 1993; Bohus et al., 1996). Interestingly, we found that CRH mRNA increased in the central amygdala (CNA) 24 h after SD in mice (Beaulieu et al., 1997). CRH mRNA was increased in the paraventricular nucleus (PVN) of the hypothalamus 2,6, and 24 h after SD. In the current experiment, stress induced-CRH and dehydration-induced AVP in the psychologically stressed mice may have respectively, inhibited and activated activity and thereby opposed one another, resulting in activity levels similar to control mice. These hypotheses could be tested, by measuring *in situ* hybridization for mRNAs for AVP and CRH in the CNA, PVN, hippocampus and supraoptic nucleus.

The effects of stress and dehydration on memory were less robust than those on activity. During trial 2 of the baseline test, C57BL/6 mice performed well and spent a greater percentage of time in the novel arm and had a greater percentage of entries into the novel arm, relative to the familiar arm (see Figure 8). Following 21 days of social stress, stressed mice tended to have a longer latency to enter the novel arm (see Figure 9) and tended to spend a lower percentage of time in the novel arm (see Figure 10). However, these effects did not reach significance. Since there is individual variability in behavior, a larger sample size may be needed in order to observe an effect on memory. Surprisingly, there were no significant effects of this chronic stress exposure to a moderate stressor on behaviors measured in the resident-intruder test. The apparent lack of fear conditioning in these mice may be related to alterations in AVP. Recall, AVP prior to a stressful experience impairs memory, but AVP following a stressful experience facilitates memory (Siegfried et al., 1984). On the day after the resident-intruder test (2 days after the last stress exposure), mice were again tested for performance in the Y-maze test. There were no significant effects of stress or dehydration on maze performance 2 days following 21 days of social stress. In a previous experiment (Beaulieu et al. 1997), we observed increased CRH mRNA in the CNA 24 h post-SD, but not 2 days post-SD. The time course of changes in AVP mRNA remains to be determined. It is unclear whether testing in the resident-intruder test affected performance on the second Y-maze post-test, or whether there were carryover effects from the Y-maze test the day before, or whether the effects of social stress were transient. McEwen (1999) observed that the hippocampal changes induced by restraint stress in rats are transient, although typically the effects last five days. We plan to increase the duration of stressful exposures to direct interaction with the aggressors in attempt to induce longer lasting changes and plan to map out changes in AVP.

We also investigated whether there are delayed effects of an acute SD on memory performance in the Y-maze in mice that were group housed or individually housed. We observed a non-significant trend for SD mice to spend more time in the familiar arm and less time in the novel arm, relative to NOSD mice, two weeks following acute SD (see Figure 11). These data were analyzed by a two way ANOVA, with housing status and defeat status as independent factors. We observed a variety of effects of housing status on behaviors in the resident-intruder test.

III. We will determine whether the proposed pharmacological agents will prevent SD-induced behavioral changes. Where appropriate, plasma corticosterone was assayed as a stress index.

The enzyme N-acetylated alpha-linked acidic dipeptidase (NAALADase) converts the weakly excitatory dipeptide putative neurotransmitter N-acetylaspartylglutamate (NAAG) to the potent excitatory neurotransmitter glutamic acid (Coyle et al., 1997). High levels of glutamate are neurotoxic (reviewed in Choi, 1988). Glutamate is released in the hippocampus during stress (Moghaddam et al., 1993). As proposed, we administered the NAALADase inhibitor PMPA intracerebroventricularly via osmotic minipumps for one week prior to acute SD. In C57BL/6 mice (see Figure 12), but not in DBA/2 mice (see Figure 13), PMPA increased the percentage of mice that attacked during either SD or during the resident-intruder test. In DBA/2 mice, PMPA increased approach behavior during the resident-intruder test (see Figure 14). Other defeat-induced behaviors were not affected. The findings of PMPA effects in mice parallel experiments in which we observed that a low dose of the classic anxiolytic diazepam induced aggressive behavior in C57BL/6 and approach in DBA/2 mice. DBA/2 mice are considered a highly reactive or anxious strain (Siegfried et al., 1984). During repeated aggressive confrontation, the defensive strategy of C57BL/6 mice changes from escape to defensive upright posture, while DBA/2 mice persist in escape behavior (Kulling et al., 1987). Recall, these mice were highly active in the Y-maze test (Figure 6). Increased approach behavior induced by both diazepam and PMPA in DBA/2 mice may indicate anxiolytic activity of these drugs. Data on strain differences in the behavioral effects of PMPA were presented at the Society for Neuroscience meeting (Lumley et al 1999; see appendix). Corticosterone levels were assayed in C57BL/6 mice (Figure 15) and in DBA/2 mice (Figure 16) that received PMPA via osmotic pumps. Possibly related to the strain differences in response to PMPA, Meyerhoff et al. (1991) found that NAALADase activity levels in the CNS are significantly less in DBA/2 mice than in C57BL/6 mice. The NAALADase levels in brains of DBA/2 mice (as a percent of C57BL/6 levels) were: amygdala (50%), frontal cortex (50%), pyriform cortex (60%), hippocampus (60%) and entorhinal cortex (70%). We plan to continue to examine potential anti-stress or neuroprotective effects of glutamatergic agents.

IV. We will determine whether testing in the Y-maze will influence behavior in the resident-intruder test. We hypothesized that Y-maze testing would not affect behavior in the resident-intruder test and that it would be valid to test the same mice in both the Y-maze and in tests of territorial defense.

Due to repeated bacterial infections found in mice from the Charles River colony, we found it necessary to change our mouse supply vendor Jackson Laboratories (see section V b). Since it took months of individual housing and training for the new stimulus mice to display aggressive behavior, the effects of SD could not be examined; baseline behaviors in the Y-maze and resident-intruder test were measured. Now that we have trained aggressors, we will test whether behaviors of defeated mice from the two vendors are similar and whether the order of tests affects behavior. In the experiment reviewed in section II, we didn't observe an effect of 21 days of daily stress exposure on behavior in the resident-intruder test. However, this repeated prolonged exposure to moderate stress differs from SD, in which subjects are subjected to three 2 min bouts of very intense attacks in the home cages of three different aggressors. Whether the lack of behavioral effects in the resident-intruder test following 21 days of psychosocial stress was related to prior maze testing on the same day, or adaptation by the mice to the daily stress exposures remains to be determined. Adaptation may have blunted their fear responses in the resident-intruder test.

V. We encountered three major obstacles during the year that slightly impeded productivity.

1. Trouble-shooting Coulbourn Y-mazes. We identified a serious design flaw in the Coulbourn apparatus and had to modify the equipment purchased from Coulbourn Inst. The photocells of the system were aligned such that when a subject was in a particular arm of the maze, the computer registered incorrectly that the subject had exited the arm (see Diagram). In determining the location to place the photocells, the vendor did not include the length of the mouse's tail as part of the entire length of the mouse's body, which functionally meant that the mouse tail could simultaneously break the 2 sets of photobeams. The computer program recorded the mouse had left the maze arm, when in fact the mouse remained within the maze arm. Surveillance cameras that we purchased allowed us to ensure that the computer correctly registered the mouse's location. Initial tests revealed the design flaw in the equipment. We modified the maze so that the photocells were located further apart than the entire length of the mouse. Coulbourn Inst. designed a new program, Y2K compatible, and so far sent us two versions to implement.
2. Infected mice from Charles River. We received 3 successive batches of infected C57BL/6 mice from Charles River. The first batch arrived positive for helicobacter hepaticus and was euthanized upon arrival by the Division of Veterinary Medicine. The second batch arrived positive for the bacteria staphacoccus aureus, which is a common bacterial infection and is usually asymptomatic. Mice were asymptomatic for the first four weeks. By four weeks post-arrival, many mice began to have preputial abscesses of unknown origin. We submitted mice for necropsy (see attached necropsy report) and it was determined that these abscesses were positive for staphacoccus aureus. Mice were euthanized and we placed another order, requesting staphacoccus aureus-free mice. Weeks after the third batch arrived, these mice too began to display preputial abscess, which were again positive for staphacoccus aureus. We euthanized our entire colony of aggressors and stimulus mice and switched vendors to Jackson Labs, who guaranteed that their mice were staphacoccus-free. In order not to contaminate new mice, we euthanized our old aggressors, which had contracted the bacteria from the infected mice. Months were required for isolation, selection and training of new aggressors.
3. Relocation of WRAIR. In Nov. and Dec. 1999, the Division of Neuroscience relocated to building 503. The expected move date changed numerous times, impacting experimental planning, although we did not completely shut down testing until just prior to actual move. Problems with the new building meeting the standards of the Laboratory Animal Care and Use Committee at WRAIR delayed relocation of animals to the Building 503 by months.

VI. KEY RESEARCH ACCOMPLISHMENTS

- The mouse strain C57BL/6 performs better on the Y-maze test than the DBA/2 mouse strain.
- With repeated daily tests in the Y-maze, exploratory activity in DBA/2 mice tends to habituate.
- Daily defeats in mice induce immobility and failure to explore in the Y-maze.
- Twenty-one successive days of 6 h psychosocial stress and food and water deprivation, incurs transiently daily loss of over 10% of body weight during the 6 h stress period and tends to impair short-term spatial memory in the Y-maze test.
- Six h food and water deprivation without social stress induces weight loss of over 5% of body weight, and significantly increases activity in the Y-maze 24 h after the last deprivation period. Both effects are transient. We suggest that dehydration may have been an unsuspected confound in experiments previously published by others.
- Chronic inhibition of NAALADase, the enzyme that converts NAAG to glutamate, induced aggressive behavior in C57BL/6 mice and increased approach behavior in DBA/2 mice, suggesting an anxiolytic effect.
- Strain differences were found in response to the NAALADase-inhibiting drug.

REPORTABLE OUTCOMES:

Manuscripts:

Lumley LA, Sipos ML, Charles RC, Charles RF, Meyerhoff JL (1999) Social stress effects on territorial marking and ultrasonic vocalizations in mice. Physiology & Behavior, 67:769-775.

Manuscript in preparation:

Lumley LA, Charles RF, Charles RC, Hebert MA, Morton DM, Meyerhoff JL. Effects of social defeat and of diazepam on behavior in a resident-intruder test in male DBA/2 mice. Submitted to Pharmacology Biochemistry and Behavior.

Lumley LA, Slusher BS, Morton DM, Charles RC, Charles RF, Saviolakis GA, Meyerhoff. Strain differences between C57BL/6 and DBA/2 mice in effects of NAALADase inhibition on aggressive behavior (in preparation).

Abstracts:

Lumley LA, Slusher BS, Morton DM, Charles RC, Charles RF, Saviolakis GA, Meyerhoff JL (1999) Behavioral effects of NAALADase inhibition in socially defeated male mice. Society for Neuroscience Abstracts, vol. 25: 59.

Lumley LA, Morton DM, Saviolakis GA, Sipos ML, Morton SJ, Meyerhoff JL (1999). Effects of social defeat on territorial urine marking in C57BL/6 mice in response to male and female stimulus mice, International Society for Psychoneuroendocrinology.

Presentations:

Lumley LA, Slusher BS, Morton DM, Charles RC, Charles RF, Saviolakis GA, Meyerhoff JL. Behavioral effects of NAALADase inhibition in socially defeated male mice. 29th Meeting of the Society for Neuroscience, Miami, FL, Oct 23-28, 1999.

Lumley LA, Morton DM, Saviolakis GA, Sipos ML, Morton SJ, Meyerhoff JL (1999). Effects of social defeat on territorial urine marking in C57BL/6 mice in response to male and female stimulus mice, International Society for Psychoneuroendocrinology, Orlando, FL.

CONCLUSIONS

We determined that C57BL/6 mice are a suitable strain to test effects of chronic psychosocial stress on spatial memory and on the integrity of the hippocampus. Daily defeats induced inactivity and lack of exploratory activity in the Y-maze, impeding measurement of memory effects in the Y-maze. In addition, levels of activity in the Y-maze tended to habituate with daily testing. With daily testing in the Y-mazes, there was high variability between test days that may in part have been caused by an innate turning bias in the mice, as the novel arm of the maze was alternated each day. We designed an experimental paradigm that would mimic the 21 days of restraint stress, which in rats and mice induced memory impairment and hippocampal changes (Magarinos and McEwen, 1995; Magarinos and McEwen, unpublished data), yet would not inhibit exploratory activity. The 21-day psychosocial stress included 6 h food and water deprivation, as occurred with the restraint stress. Mice exposed to an aggressor within an enclosure in the aggressor's home cage for 21 consecutive days for 6 h/day displayed robust but transient weight loss during each daily deprivation period (body weight recovered before the next test), and tended to be impaired in the Y-maze test. However, these mice were not inactive during the Y-maze or during the modified resident intruder test. We plan to use this mouse model to examine stress-induced changes in the hippocampus and to screen potential neuroprotective agents. Since many effects of repeated social stress on memory impairment were non-significant trends and the effects were transient, we plan to increase the duration and frequency of stressful direct interactive agonistic encounters with the aggressors during the 6 h stress periods. In addition, we plan to use larger sample sizes, since there is high individual variability in behaviors.

We also tested whether chronic exposure (intracerebroventricularly) to a NAALADase inhibitor would prevent or reverse effects of acute SD (see attached abstract and Figures 13-16). In C57BL/6 mice, NAADADase inhibition induced aggressive behavior, whereas in DBA/2 mice, NAALADase inhibition increased approach behavior. NAALADase inhibition prevents the conversion of NAAG to glutamate (ie. Jackson et al 1996). The effects on behavior of NAALADase inhibition may be via direct effects of NAAG, which acts as an NMDA antagonist and as a metabotropic glutamate agonist. Antagonists at the NMDA receptor have been reported to increase aggressive behavior (Belozertseva et al 1999). Alternatively, the effects may be through decreased levels of glutamate at discrete sites. Whether NAALADase inhibition also prevents stress-induced memory and hippocampal changes remains to be determined.

FUTURE DIRECTIONS

We plan to continue to examine effects of chronic social stress on memory impairment in order to determine the time course of stress exposure necessary to induce memory impairment. We plan to include a Morris water maze test in order to control for potential effects of social stress on immobility in a test of exploration. After we determine the extent and duration of social stress exposures that impair memory, we will examine the hippocampus for markers of neurochemical and morphological changes. It is possible that the reported increase in density of mitochondria in apical hippocampal pyramidal dendrites (Magarinos et al., 1997) may be due to the dehydration or other nutritive factors inadvertently associated with the restraint stress paradigm.

In soldiers, dehydration rapidly causes battle fatigue (see Jones et al., 1995) and in mice, dehydration affects performance in the Y-maze (see section 2). Since dehydration increases AVP (Oliet et al. 1994), and since AVP affects memory (Koob et al. 1985), we plan to examine CNS AVP levels in areas that are implicated in stress and memory, including the hippocampus, paraventricular area, supraoptic area, and the amygdala.

We plan to continue to examine glutamatergic ligands for their potential in preventing or reversing stress-induced changes. The model appears to be well-suited as a screen for potential anxiolytic drugs.

Finally, strain differences may be a very productive dimension to explore (e.g. the hypercortisolemic strain reported by Barden (Pepin et al., 1992). This model is suitable for exploration of genetically-determined vulnerability vs. resilience to stress.

Barden's group has expressed an interest in collaborating with us.

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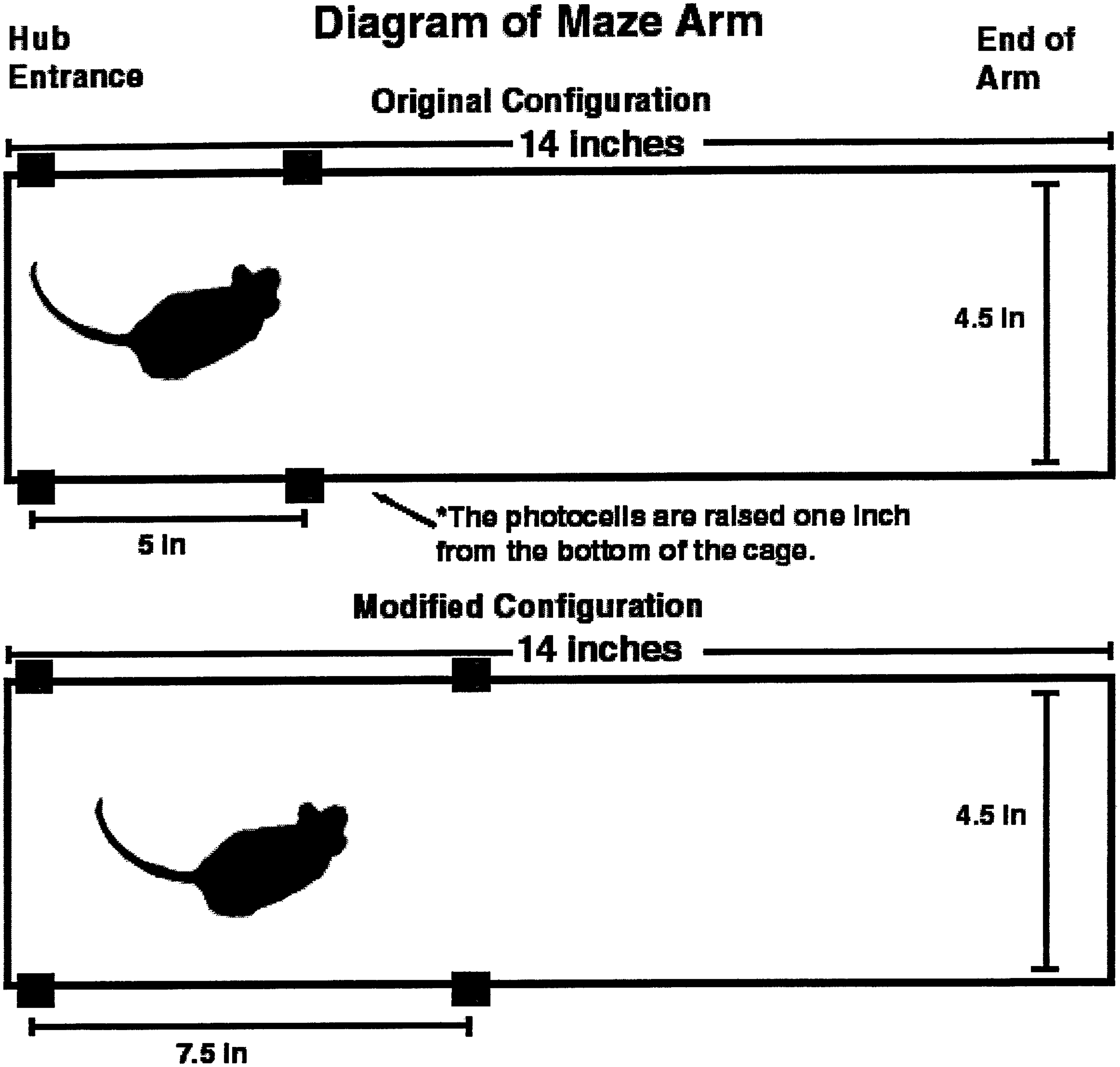
Diagram of Maze Arm



The design of the Y-maze runway by Coulbourn Instruments places the second set of photocells a body length away from the first set. This design fails to take into account the tail of the animal, which is capable of breaking the photo-beam. In order to correct this flaw the second set of photocells must be placed two-thirds down the runway.

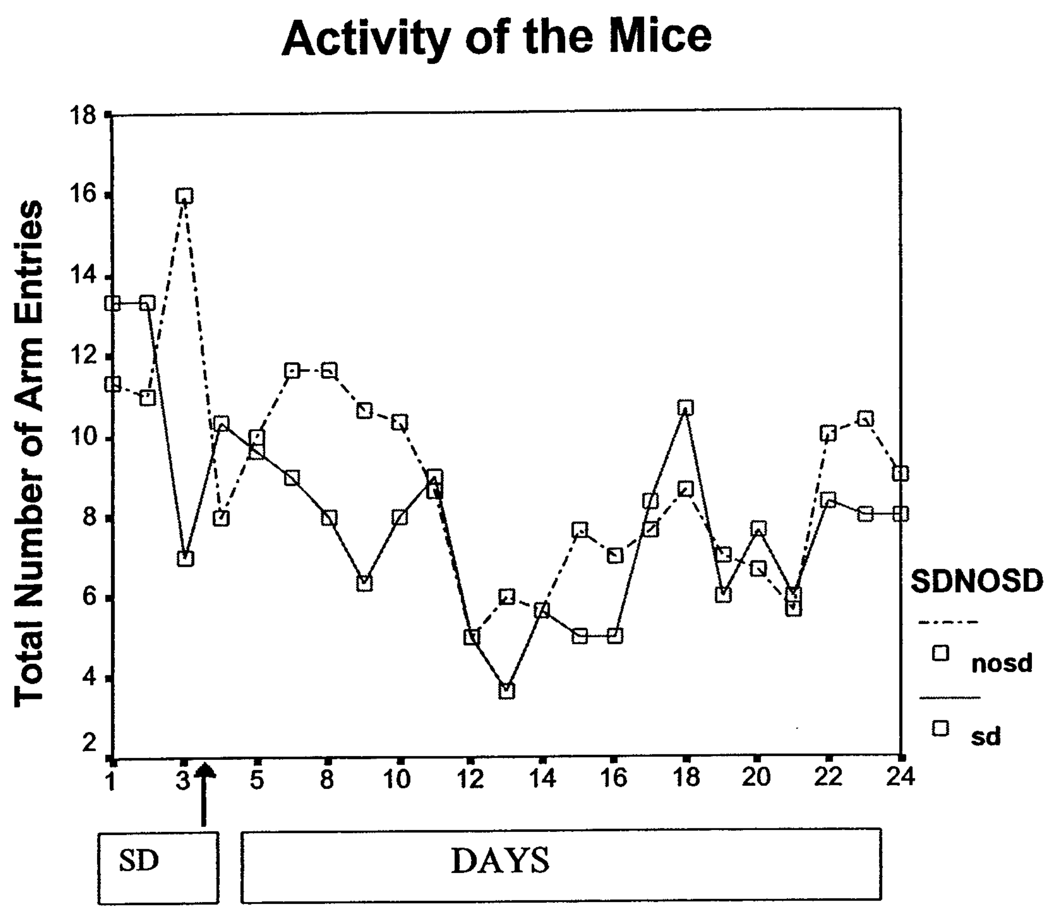


Figure 1. Baseline tests were run on Day 1 through Day 3. Subjects were defeated daily 2 to 4 hrs after the maze experiment on Day 3 through Day 20. The total number of entries into the arms of the maze is a measure of the overall activity of the subject. The number of entries for both the SD and NOSD group decreased as the days progressed. This is possibly a result of the habituation of the mice to the maze, which may mask the effects of defeat on spatial memory. Data from Day 6, the third post-test, was not collected.

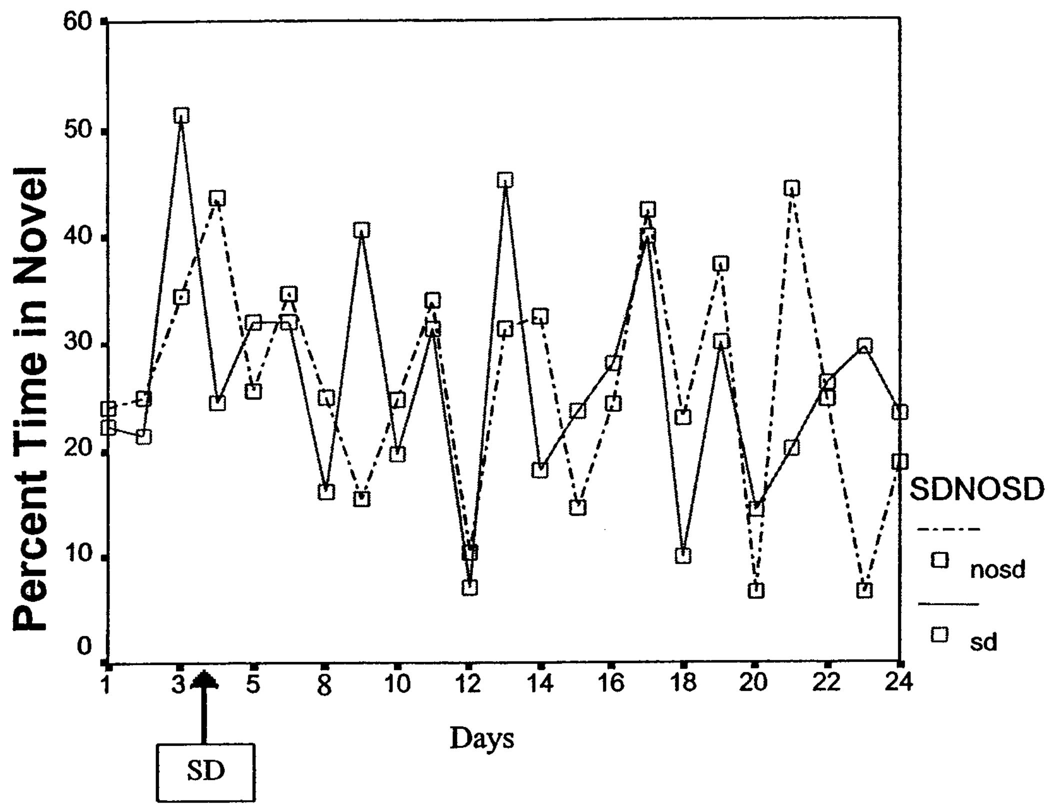


Figure 2. Both SD and NOSD DBA/2 mice tended to spend more time in the same arm of the maze, regardless of whether it was the novel or the familiar arm of the maze. This finding may indicate a turning preference, since the novel and familiar arms were alternated daily. Although the mice were tested on day 6 (post-test 3), data is missing from this day due to equipment failure.

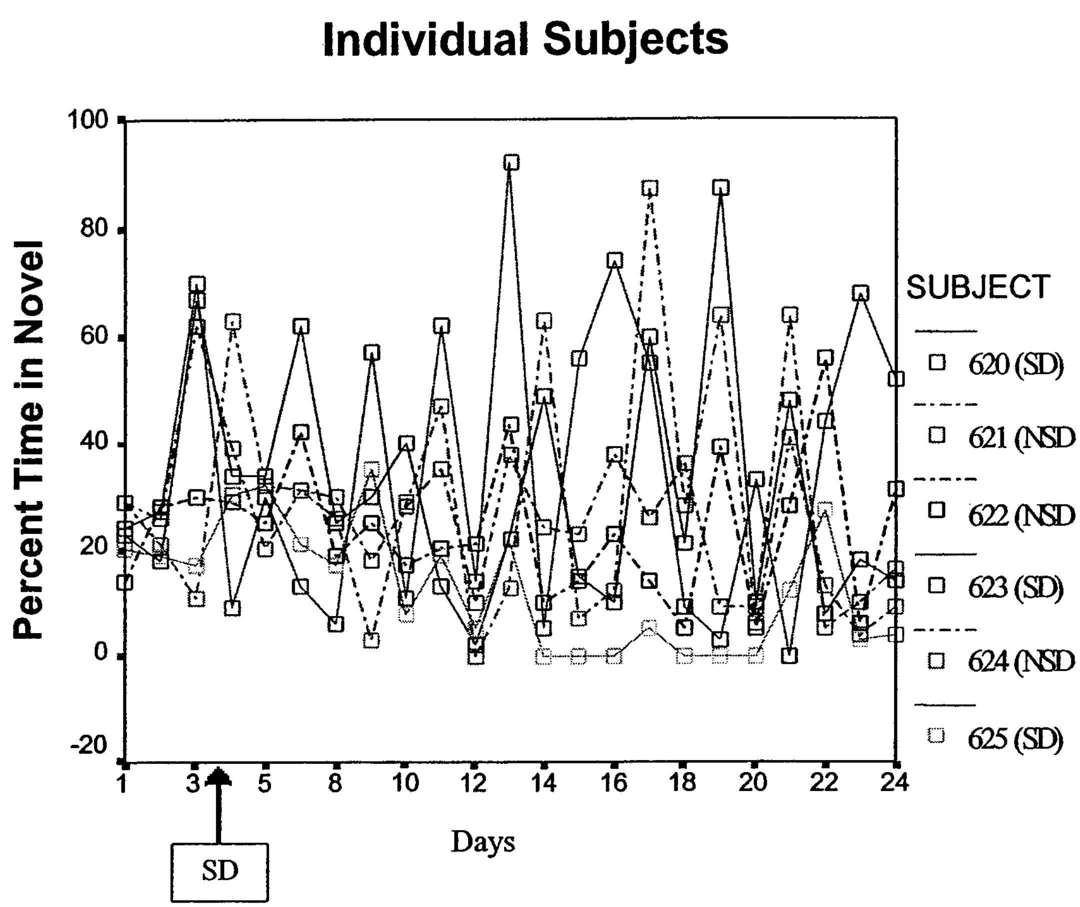


Figure 3. The DBA/2 mice displayed high variability in the percentage of total time spent in the novel arm. No significant difference was observed between the NOSD and SD groups for this factor.

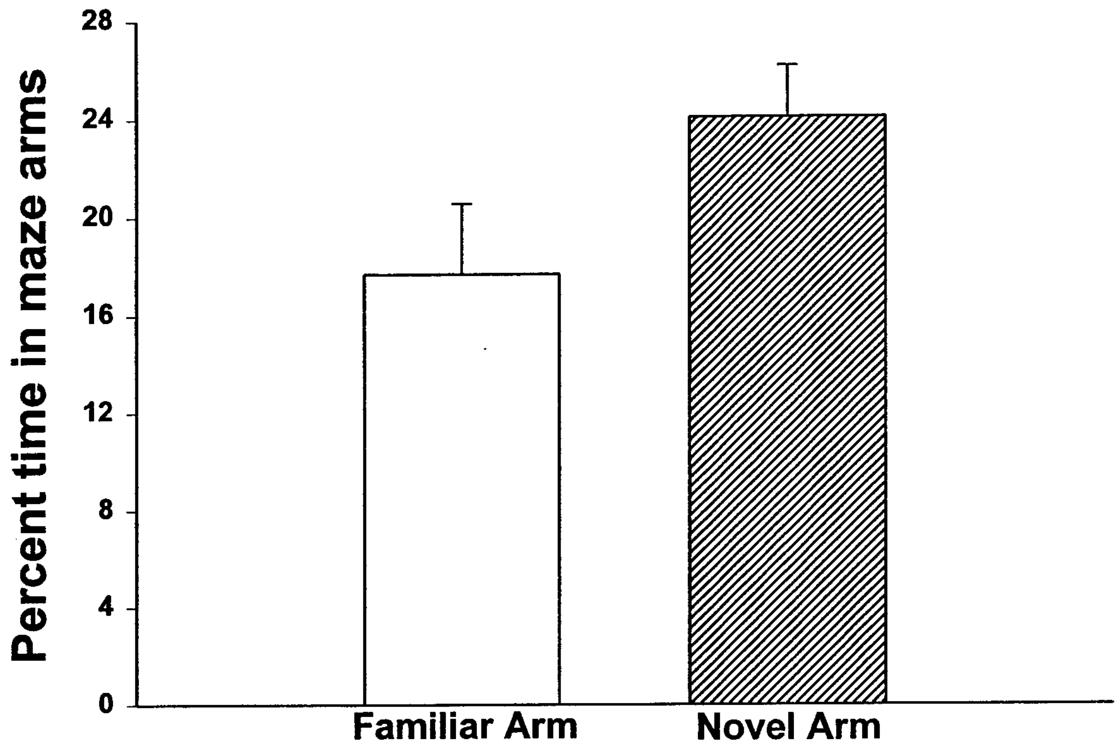


Figure 4. During trial 2 of the baseline test, there was a non-significant trend for the combined groups of C57BL/6 and DBA/2 mice to spend a greater percent of time in the novel arm than in the familiar arm, as expected.

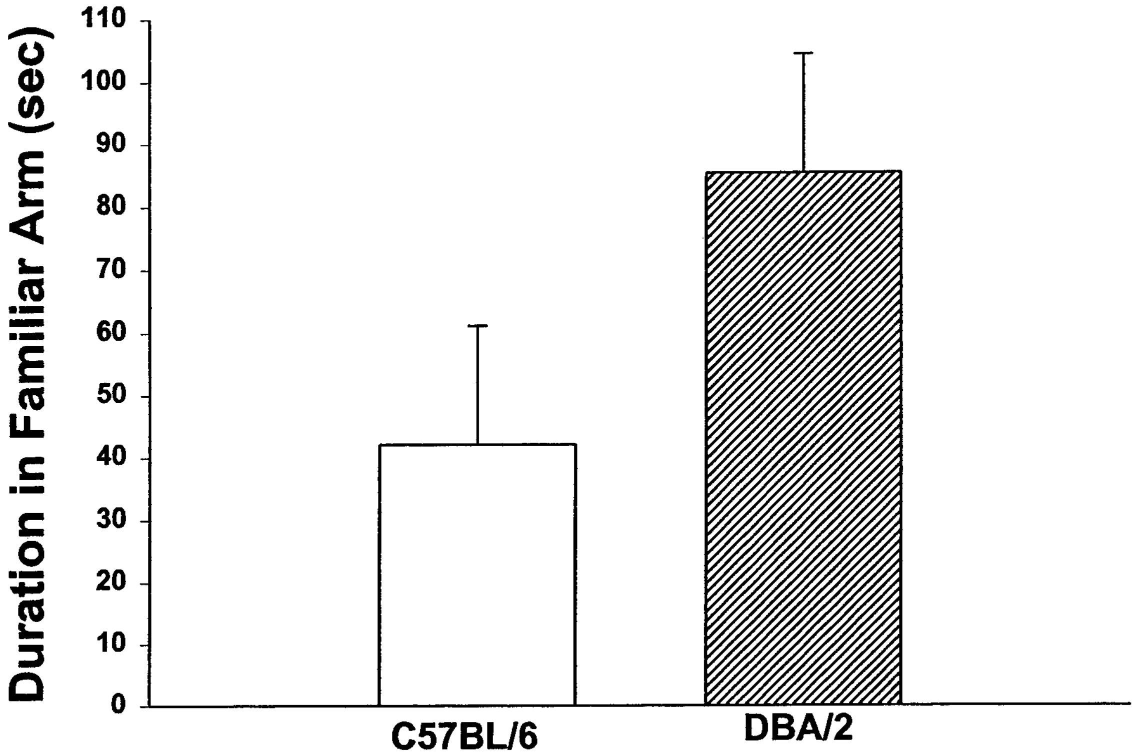


Figure 5. During trial 2 of the baseline test, male DBA/2 mice tested in the Y-maze tended to spend more time in the familiar arm than did C57BL/6 mice.

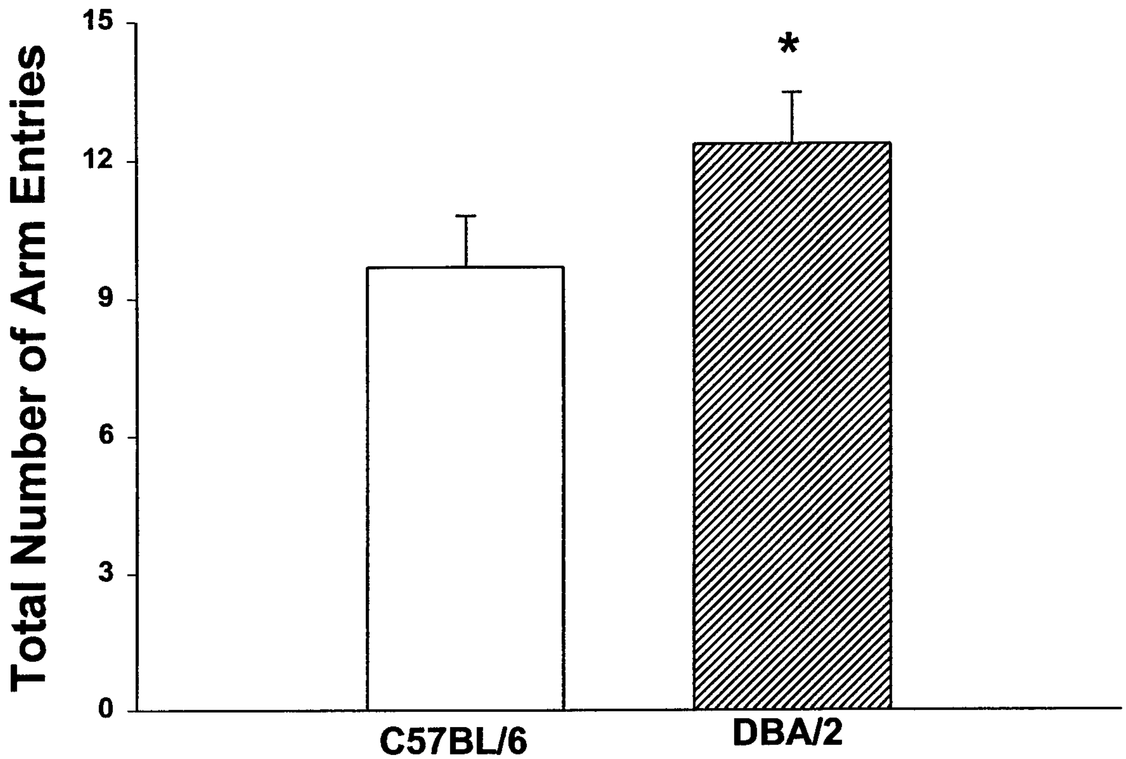


Figure 6. Male DBA/2 mice tested in the Y-maze changed arms more frequenty (more total entries) than C57BL/6 mice, during baseline one. *p<0.05

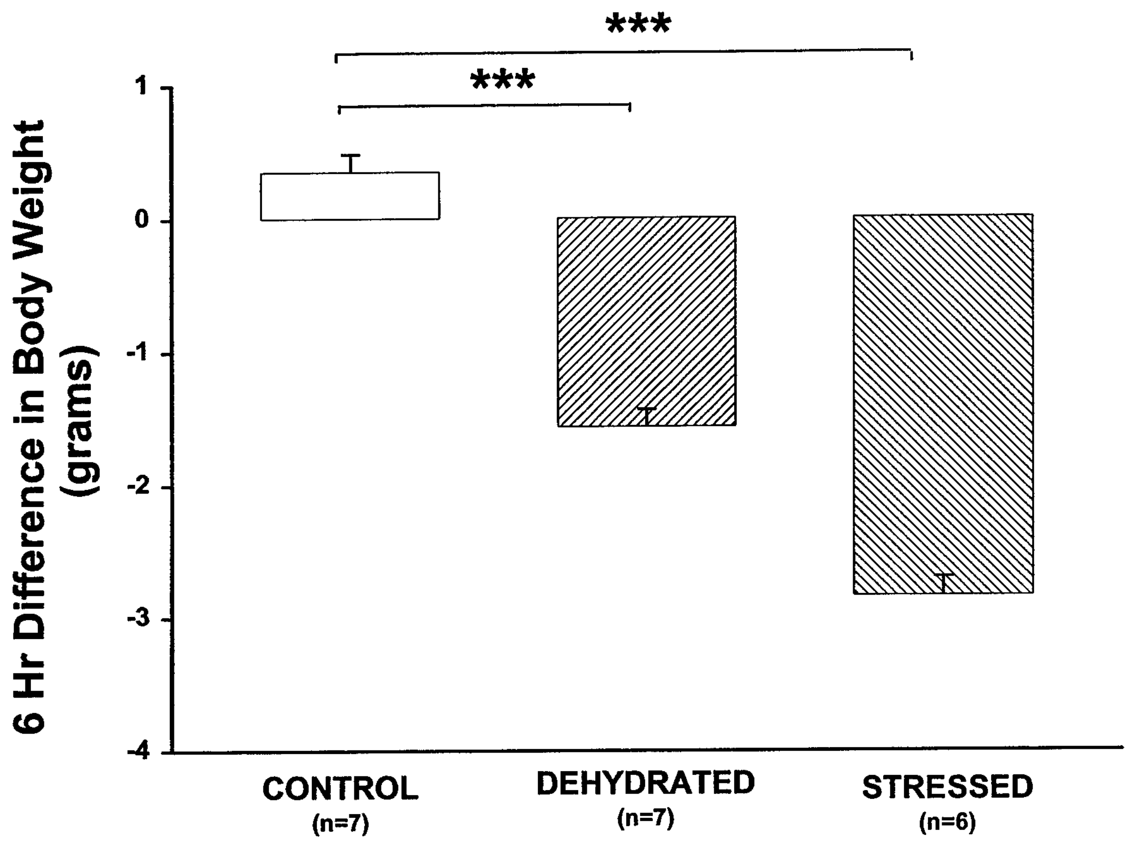


Figure 7A. C57BL/6 mice that received 21 days of social stress transiently lost more weight (over 10% body weight) during each 6 h daily stress exposure than did control mice or mice exposed to 6 h dehydration alone. Mice that received 21 days of daily 6 h dehydration alone transiently lost more weight than control mice. ***p<0.001

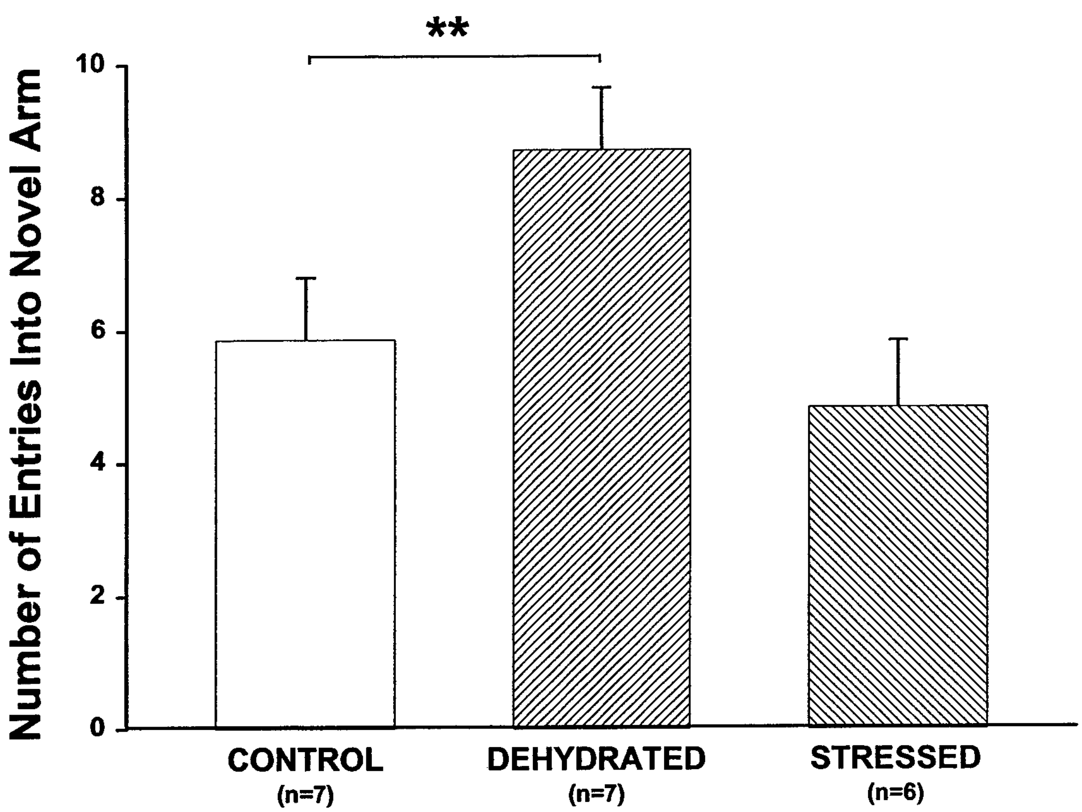


Figure 7B. C57BL/6 mice that received 21 days of 6 h food and water deprivation (DEHYDRATED) were more active in the Y-maze test than control mice, demonstrated by an increased number of entries into the novel arm. Dehydrated mice also tended to have increased entries into the familiar arm. **p<0.02

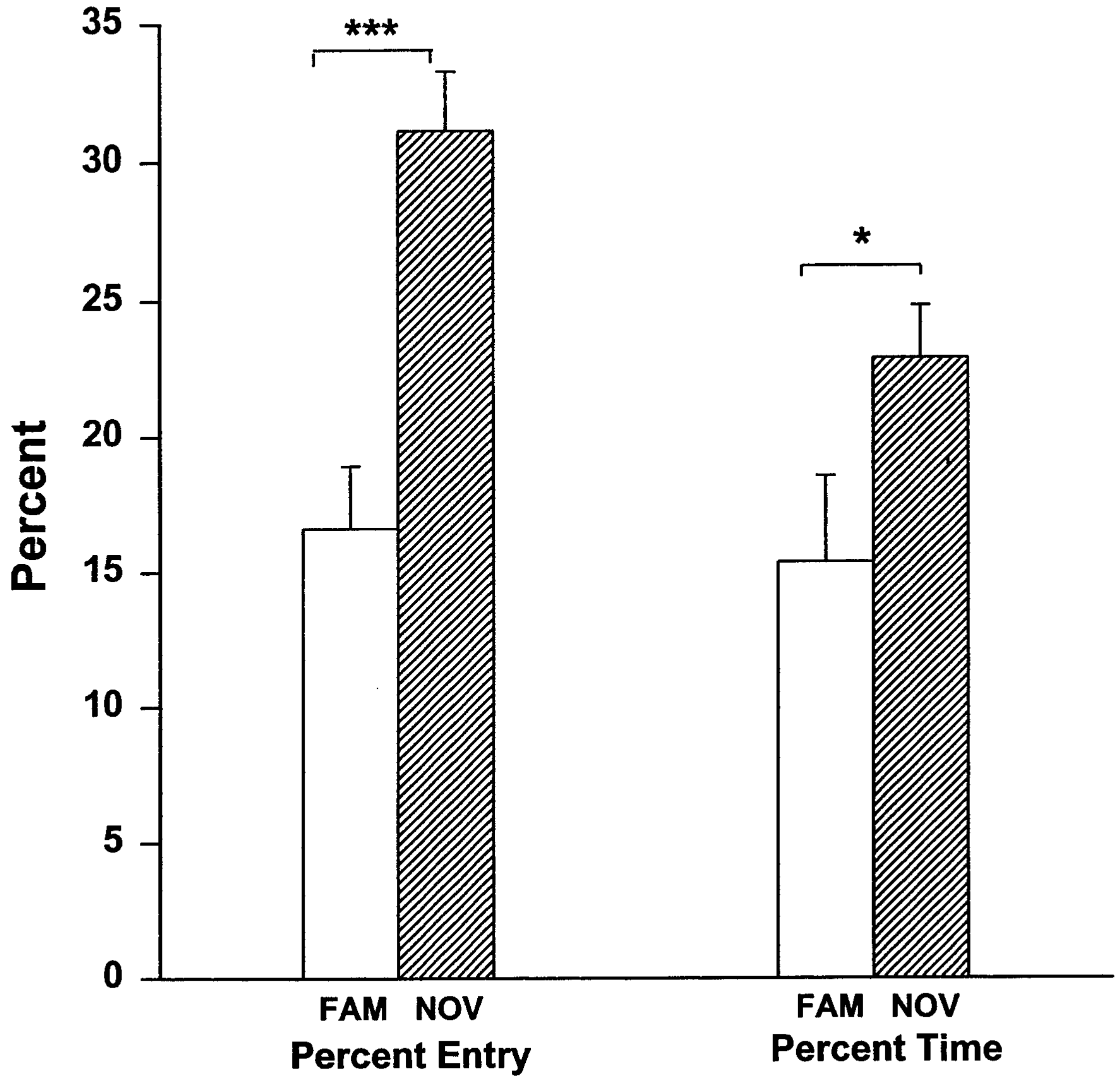


Figure 8. During baseline test, C57BL/6 mice (n=20) spent a greater percentage of time in the novel than the familiar arm, and had a greater percentage of entries into the novel arm. ***$p<0.001$ *$p<0.05$

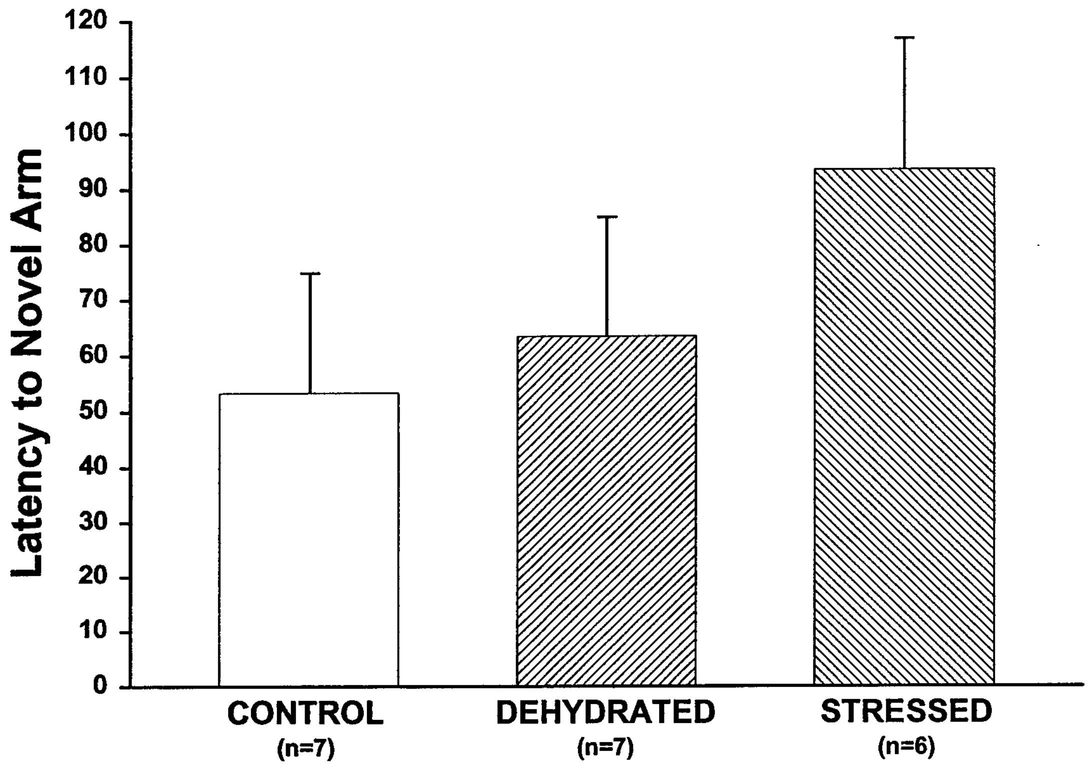


Figure 9. Socially stressed C57BL/6 mice tended to have increased latency to enter the novel arm of the Y-maze, following 21 days of 6 h stress. However, this was not significant.

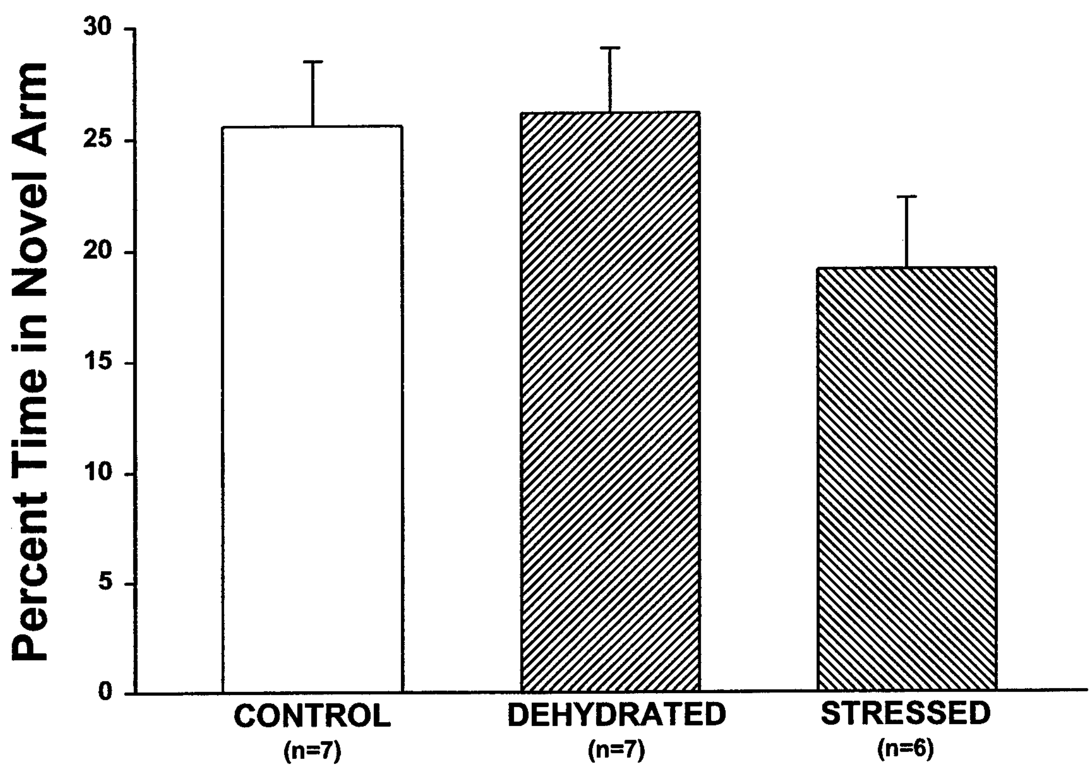


Figure 10. There was a non-significant trend for socially stressed C57BL/6 mice to spend a lower percentage of time in the novel arm of the Y maze, following 21 days of 6 h stress.

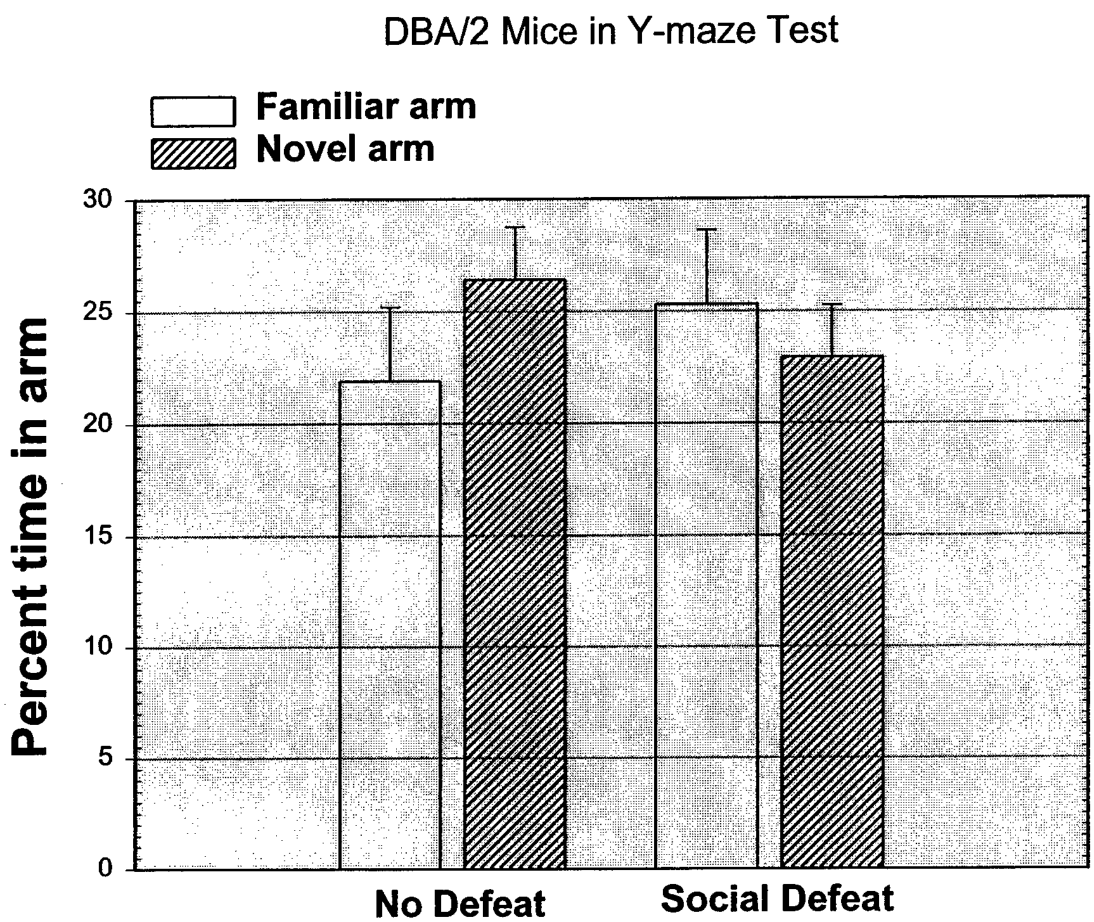


Figure 11. There were non-significant trends for mice that were defeated 2 weeks prior to a Y--maze test of short term spatial memory to spend a greater percent of time in the familiar arm than did non-defeated mice, during trial 2.

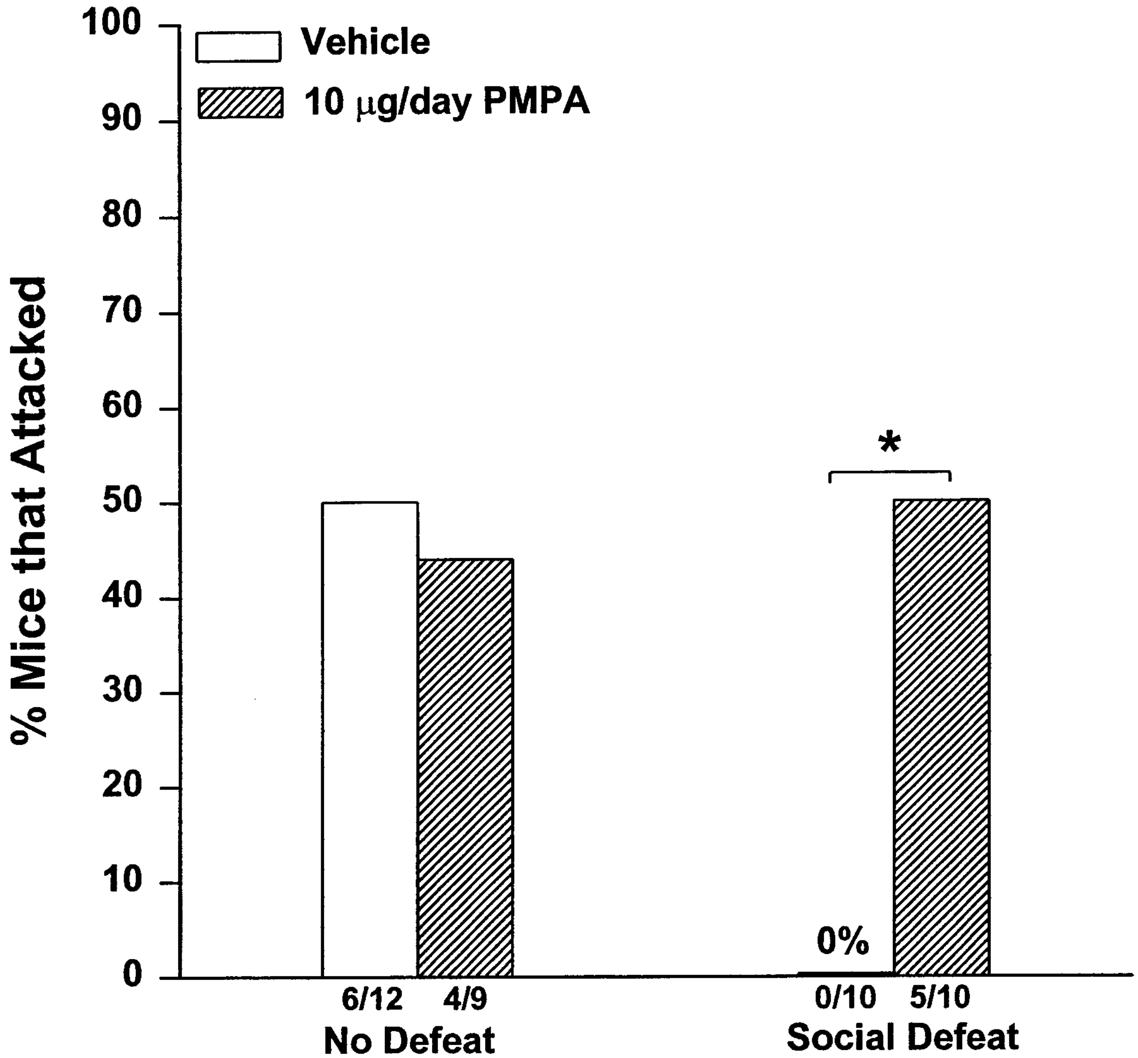


Figure 12. A greater percentage of SD C57BL/6 mice that received 2-PMPA (10 µg/day, i.c.v.) attacked either an aggressor during SD or a non-aggressor during the resident-intruder test than those that received vehicle. *p<0.05

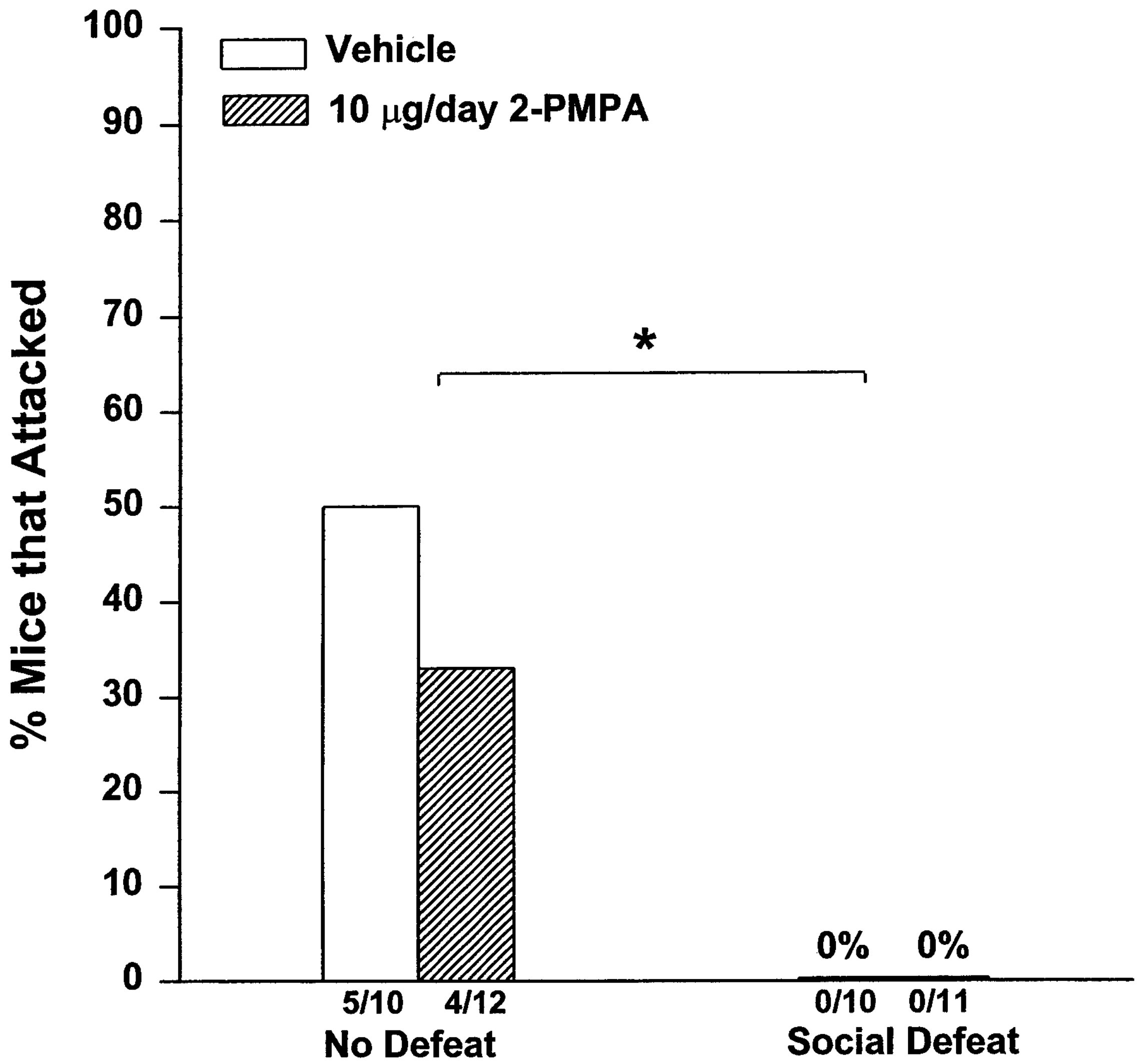


Figure 13. Only DBA/2 mice that were not defeated attacked the non-aggressive intruder during the resident intruder test (*p<0.01). There was no effect of 2-PMPA treatment (10 μg/day, i.c.v.).

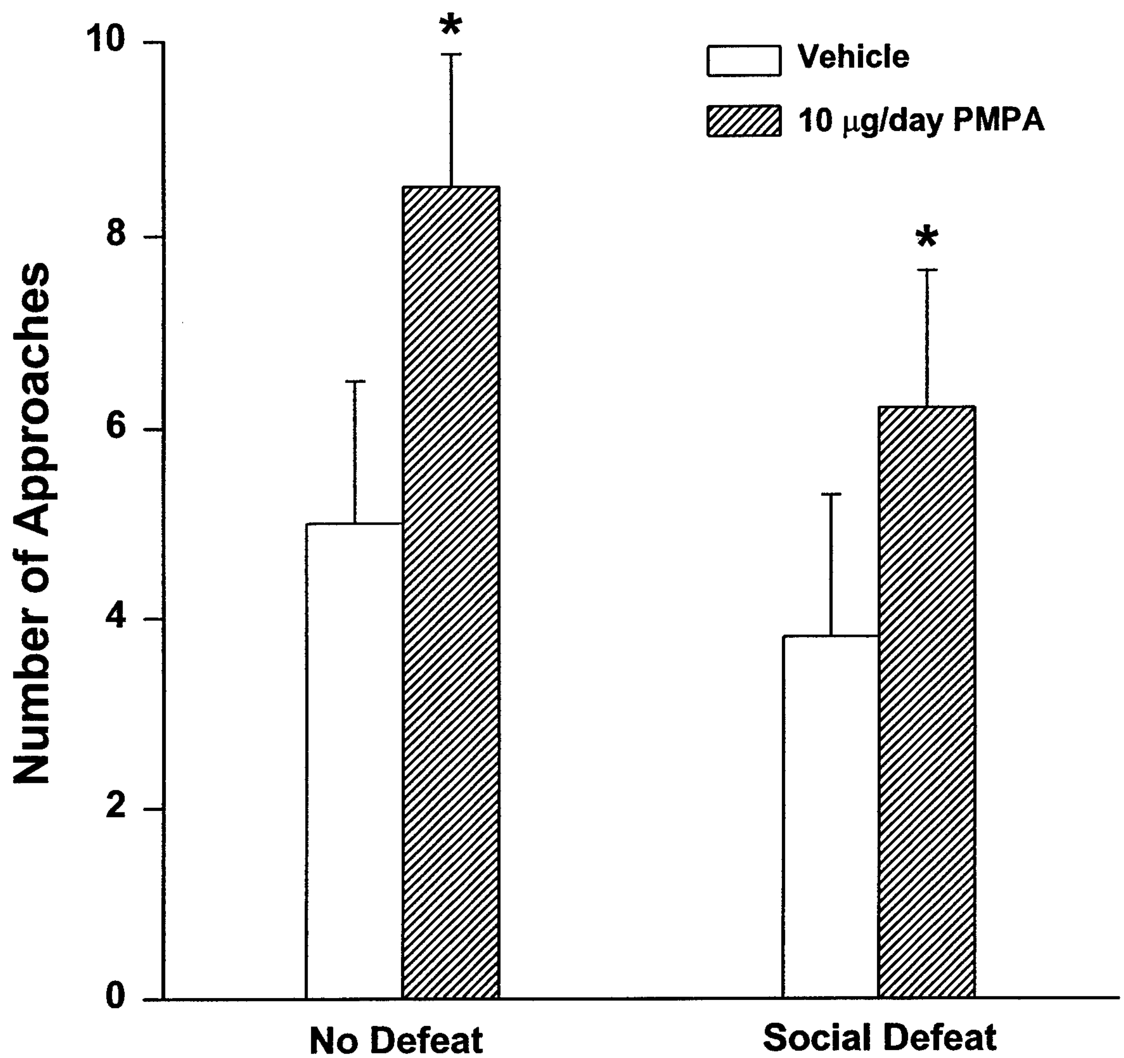


Figure 14. DBA/2 resident subject mice that received 2-PMPA (10 μg/day, i.c.v.) displayed increased approach towards a non-aggressive intruder during the barrier part of the resident intruder test. *p<0.05

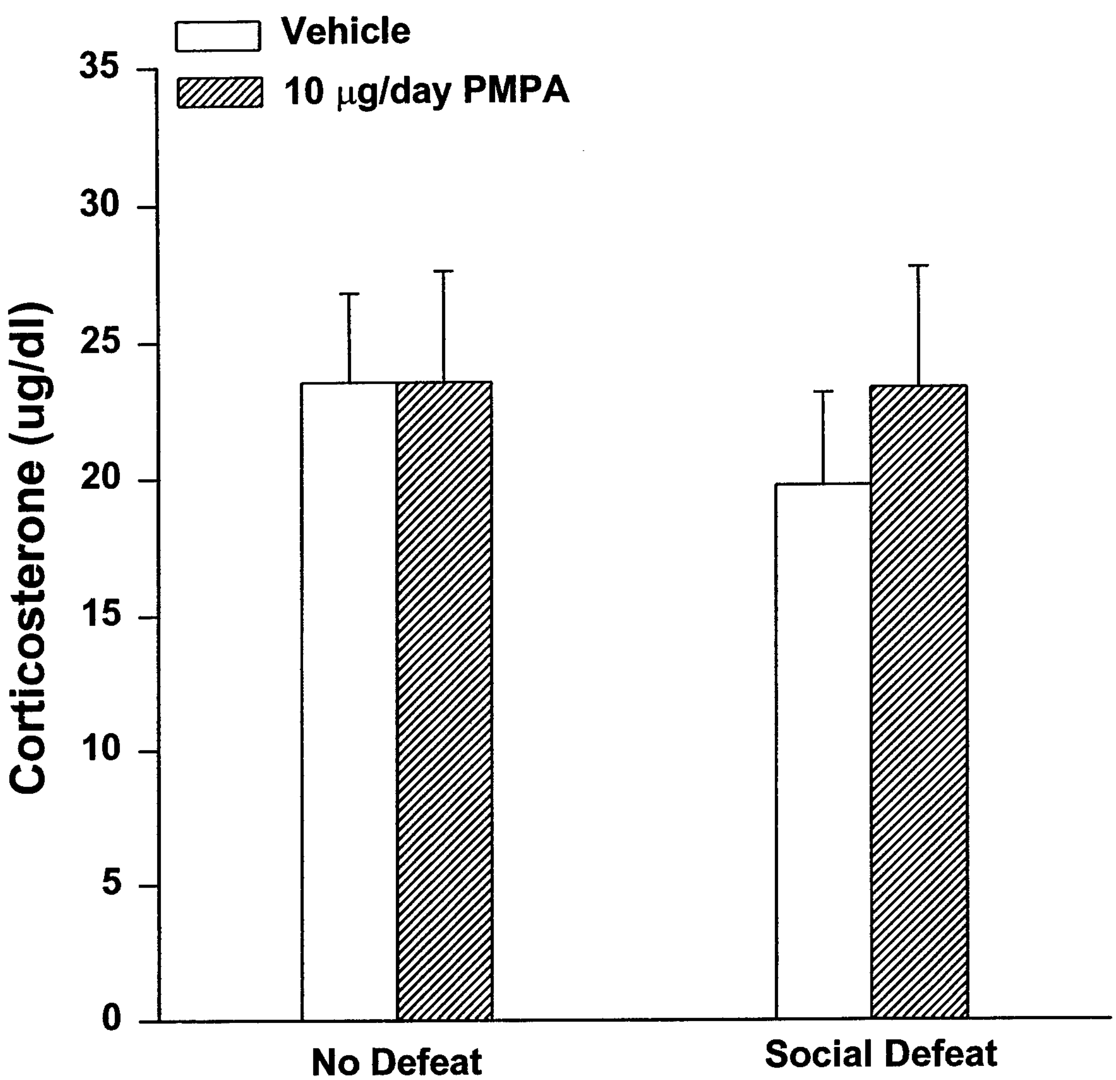


Figure 15. There was no significant effect of social defeat or of 2-PMPA (10 ug/day, icv) on plasma corticosterone levels in C57BL/6 mice.

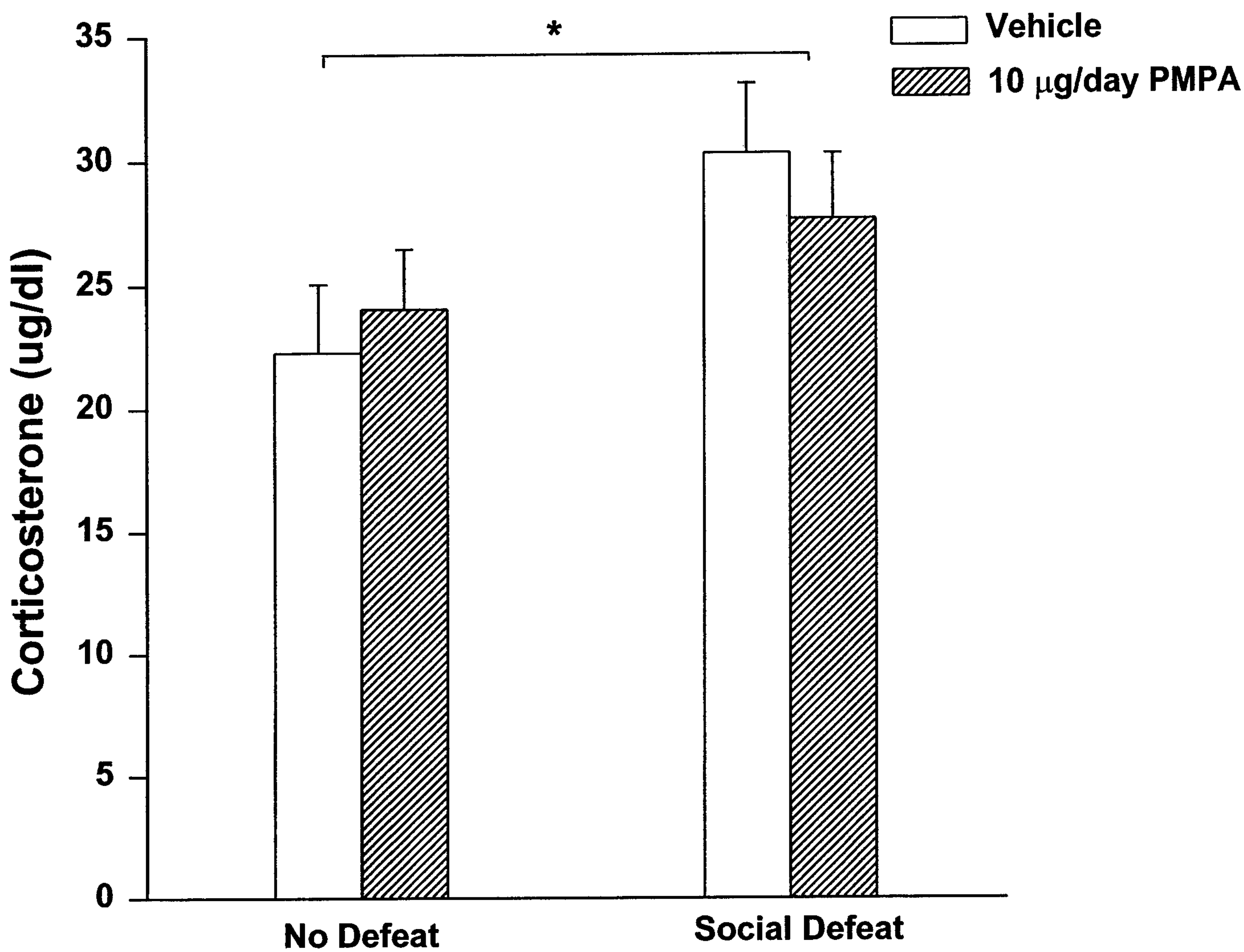


Figure 16. In DBA/2 mice, socially defeated mice had greater plasma corticosterone than had non-defeated mice (*p<0.04). There was no effect of 2-PMPA.

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Social Stress Effects on Territorial Marking and Ultrasonic Vocalizations in Mice

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LUMLEY, L. A., M. L. SIPOS, R. C. CHARLES, R. F. CHARLES AND J. L. MEYERHOFF. *Effects of social stress on territorial marking and vocalizations in male mice.* PHYSIOL BEHAV **67**(5) 769–775, 1999.—Acute social defeat (SD) leads to transient and persistent physiological and behavioral changes. We examined the effects of acute SD on territorial urine marking and ultrasonic courtship vocalizations in DBA/2 male mice. Both behaviors are considered androgen dependent and are influenced by social status, with dominant mice displaying more of both behaviors. In Experiment 1, male mice that received SD displayed prolonged inhibition of territorial urine marking, relative to nondefeated control mice (NOSD). In addition, territorial marking increased with repeated tests. In Experiment 2, male mice that received 3 successive days of SD displayed fewer ultrasonic courtship vocalizations at 30 min. post-SD1 and 30 min. post-SD2, relative to NOSD mice. In Experiment 2, we also observed decreased territorial marking 4 weeks post-SD. In sum, SD induced prolonged inhibition of territorial marking, but had only transient effects on ultrasonic courtship vocalizations, suggesting that different mechanisms may mediate the maintenance of these behaviors. © 1999 Elsevier Science Inc.

Stress Social stress Mice Territorial urine marking Ultrasonic vocalizations Defeat Urine Territorial behavior

AGONISTIC encounters are important in establishing social hierarchies in mice [(11); reviewed in (58)]. Such aggressive encounters are stressful, and have been used to study the effects of stress on physiological, hormonal, neurochemical, and behavioral measures in attempts to model a variety of stress-related disorders. The time course of changes is highly variable, depending on what is measured. In mice, some social stress-related changes are transient, such as increased swim immobility (22), analgesia (42,43,50), elevated opioid levels (51), and increased corticosterone (34). Other changes are prolonged, such as avoidance of nonaggressive intruders (20,21,34), or delayed in appearance, such as increased sensitivity to cocaine (41,44). Differences in the time course of behavioral, biochemical, and/or physiological changes following acute social defeat (SD) may help elucidate the mechanisms of acute stress disorders.

Two androgen-dependent reproductive behaviors influenced by dominance status are territorial urine marking and ultrasonic vocalizations to females (16,27,45,47,48,52). Territorial urine marking is considered an adaptive behavior used by males to signal females, as a primer for reproduction, to communicate to male intruder mice to avoid their territory, and to send alarm signals [(9,16,48); see (52)]. Dominant males mark more than subordinate males (9,16). In addition, repeated social defeats decrease male mouse sexual behavior (61), and socially subordinate males have decreased sexual motivation (14). For example, subordinate male mice display fewer 70-kHz ultrasonic courtship vocalizations during sexually motivated encounters with female mice (47).

In our laboratory, we have used acute SD in mice as a model of acute stress disorder. Socially defeated animals tend to display behavioral profiles similar to those seen in subordinate animals. Because social status influences reproduction and is altered by defeat, we hypothesized that several naturally occurring behaviors related to reproduction would also be affected by SD. In particular, we hypothesized that SD would significantly reduce the normally high level of territorial urine marking and courtship vocalizations. In addition, we were interested in determining the time course of recovery of these behaviors in SD mice. In Experiment 1, the territorial urine marking in socially defeated male mice was compared to marking in nondefeated (NOSD) mice, and the time course for changes in marking levels was examined. In Experiment 2, we examined whether SD would affect ultrasonic vocalizations to female cage bedding, relative to NOSD mice. Our re-

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sults show that territorial urine marking and ultrasonic vocalizations decrease following SD. However, the time course of the changes in these behaviors is different; changes in urine marking are long lasting, whereas changes in ultrasonic vocalizations are transient. These experiments examine additional behavioral measures that may be useful in elucidating the mechanism of physiological and behavioral changes following SD.

GENERAL METHODS

Research was conducted in compliance with the Animal Care Welfare Act, and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles in the Guide for Care and Use of Laboratory Animals, National Research Council, National Academic Press, Washington, DC, 1996.

Animals

Adult male DBA/2 mice (20–22 g; Harlan–Sprague–Dawley) were housed in reverse 12 L:12 D cycle (lights off at 0900 h) in a temperature (20 ± 3°C)- and humidity (50 ± 20%)-controlled room, with food and water available ad lib. Subjects were initially housed five mice per cage (48 × 27 × 20 cm), and were individually housed 2 weeks prior to the beginning of the test.

Male C57BL/6 mice (25–30 g) were used as aggressors, and were individually housed for at least 2 months prior to the experiment to promote isolation-induced aggressiveness. Aggressors were selected if they had short attack latencies and initiated many attacks within a short period of time. Aggressiveness was increased by pairing C57BL/6 mice with previously defeated DBA/2 mice twice per week.

Five males and five females DBA/2 mice (18–20 g) were used as stimulus mice for the social experience tests. Fresh cage bedding from five group-housed female mice was used in Experiment 2. The estrous cycle of the female stimulus mouse was not monitored, because female mouse urine is sexually arousing to male mice at all points in the cycle (16).

Acute Social Defeat (SD)

Social defeat consisted of three 2-min agonistic encounters with trained aggressors. Defeated mice were placed in the home cages of three successive aggressors. Subjects were separated from the aggressor for 2 min between each trial by placing a barrier within the aggressor's cage. During SD, the number of attacks and bites were recorded with a hand-held counter. Mice received 30 bites/trial during these sessions.

EXPERIMENT 1

Materials and Methods

Procedure. Because there is individual variability in territorial urine marking, subjects were prescreened for baseline urine marking. Ten of 15 mice displayed high marking levels during a baseline test, and were pair matched with another male with similar marking levels. The other five mice were excluded from the experiment. One member of each of the five subject pairs received SD. The other members of each pair did not receive SD, and served as control mice (NOSD). Twenty-four hours following SD or NOSD, matched pairs were tested for territorial urine marking, and were retested once per week for 5 weeks. In the sixth week, the mice that had previously been defeated received a second SD. No additional defeats occurred prior to the sixth week.

Territorial urine marking. Matched pairs, separated by a perforated barrier, were placed in a bottomless Plexiglas cage on Whatman Benchkote filter paper for 15 min. The barrier prevented direct physical contact, but allowed olfactory, visual, and auditory cues to be received. Mouse urine fluoresces under ultraviolet light and can be easily quantified (16). The number of urine marks was measured by overlapping a transparent grid over the filter paper under a 15-W ultraviolet light (360 nm) in a dark room and counting the number of grids (12 × 12 mm) containing urine marks. The number of grids containing urine marks and the actual number of urine marks present has been found be highly correlated [$r = 0.97$, $p < 0.01$ (35,49)]. Pools of urine larger than four square grids that formed a larger quadrant were not included in the quantification. Four squares in a row, however, were included.

Statistical analysis. The statistics program SPSS 9.0 was used to do the statistical analyses. A repeated-measures (trial) analysis of variance (ANOVA) with one between factor (defeat or no defeat) was used to determine whether there were any significant differences in territorial urine marking. This analysis included power analysis and a Mauchley's test of sphericity, which is used to verify the variance–covariance matrix structure of the dependent variable. If sphericity was rejected, the appropriate adjustment in the degrees of freedom was made using Huynh–Feldt epsilon to make the statistical analyses more conservative. Data were further probed using the Least significance difference t-test.

Results

During the second SD, one previously defeated mouse attacked the aggressors and was not defeated. Therefore, the final territorial marking score for this mouse was omitted from data analysis, leaving $n = 4$ in this group of mice. To do a repeated-measures analysis using all five subjects in the SD group, a harmonic mean was determined for the missing value. There was no significant difference in baseline urine marking levels between the matched NOSD mice (mean ± SE: 99.4 ± 42.8) and the SD mice [87.0 ± 28.9; $t(8) = 0.24$, $p = 0.82$). Because Mauchly's test of sphericity was significant, and because sphericity was rejected, the Huynh–Feldt (HF) epsilon was used to adjust the degrees of freedom, making the test more conservative. Figure 1 illustrates the means of territorial urine marking over repeated tests in defeated and nondefeated mice. Mice that received acute SD displayed less territorial urine marking than did their matched, NOSD controls, $F(1, 8) = 6.74$, $p = 0.03$; using $\alpha = 0.05$, the power was equal to 0.63. Overall, urine marking levels increased with repeated testing ($F = 4.72$, HF adjusted $df = 3.54$, $p = 0.006$, power $= 0.89$). Pair-wise comparisons revealed that mice marked more on week 4 relative to week 1 ($p < 0.03$) and 2 ($p < 0.02$), week 5 relative to weeks 1 ($p < 0.03$) and 2 ($p < 0.02$), and week 6 relative to weeks 1 ($p < 0.02$), 2 ($p < 0.01$) and 3 ($p < 0.05$). There was no significant interaction between defeat status and time ($F = 2.67$; HF adjusted $df = 3.54$, $p = 0.06$; power $= 0.64$). Because the power was only 0.64 at $\alpha = 0.06$, pair-wise comparisons were made between SD and NOSD at select time points, including post-SD2. SD mice marked significantly less than NOSD mice 5 weeks post-SD, $t(8) = 3.11$, $p < 0.02$, and tended to mark less 2 weeks post-SD, $t(8) = 7.60$, $p < 0.09$, and 4 weeks post-SD, $t(8) = 7.97$, $p = 0.07$. Twenty-four hours following a second defeat, SD mice marked significantly less than NOSD mice, $t(8) = 6.93$, $p < 0.001$.

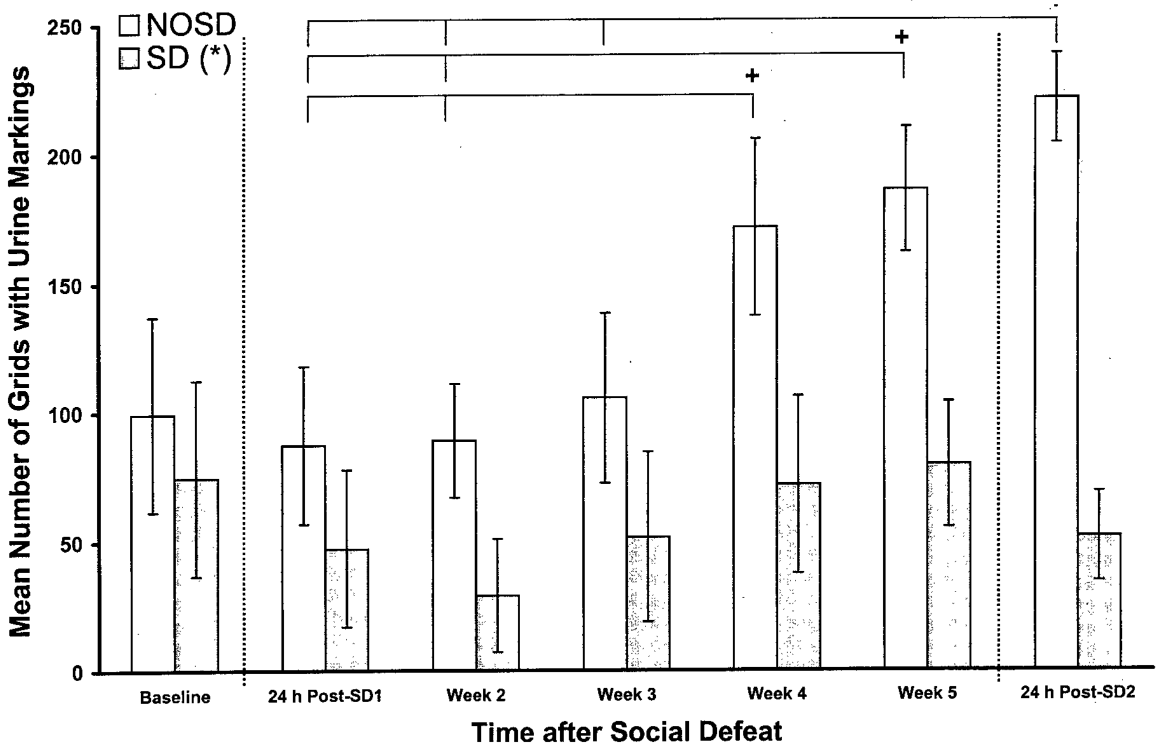

FIG. 1. Experiment 1: SD mice displayed less marking than did nondefeated (NOSD) mice (*$p = 0.03$). In addition, there was increased marking with repeated tests ($p = 0.006$). Mice marked more on Weeks 4 and 5 relative to Weeks 1 and 2, and more on Week 6 relative to Weeks 1, 2, and 3 (significant differences are shown in brackets; $+p < .05$).

EXPERIMENT 2

Materials and Methods

Procedure. Mice received 8 days of social experience and were prescreened for ultrasonic courtship vocalizations. As in Experiment 1, mice ($n = 22$) were pair matched using their baseline territorial urine marking as matching criterion. Eleven mice received repeated SD, and 11 mice were not exposed to SD. SD mice received 3 successive days of SD, and were tested for ultrasonic courtship vocalizations to female cage bedding 30-min post-SD on each of the 3 days. In addition, mice were tested for ultrasonic vocalizations 24 and 72 h following the third SD or no-SD. Mice were tested 4 weeks post-SD for territorial urine marking.

Social experience. During each of 8 days of social experience, subjects sequentially encountered a nonaggressive male and a female for 3 min each to gain social experience. The order in which the male and female mice were presented during social experience was alternated each day. During the final 2 days of social experience, vocalizations were monitored during the interaction of the subject with the female. Mice that did not vocalize during these tests or during the baseline predefeat vocalization test were omitted from the study.

Ultrasonic vocalizations to female cage bedding. Vocalizations were monitored with an ultrasonic receiver (Model U-30, Ultrasound Advice, London, UK) tuned to a center frequency of 70 kHz. Each subject was placed in a novel clean cage under the ultrasonic microphone. The cage was placed within a sound-attenuating chamber to prevent disturbance from outside noises. A 1-min habituation period without the presence of fresh female cage bedding preceded the test, to ensure that subjects did not emit random vocalizations to the test situation. If a subject vocalized during the habituation period, the period was extended until the subject ceased vocalizing for several minutes. The stimulus used to elicit vocalizations was soiled cage bedding from a cage of five group-housed females. This bedding was placed into a novel cage with the subject. The 3 min test was divided into 36 5-s time sampling intervals, during which the number of intervals containing vocalizations was recorded yielding scores ranging between 0 to 36.

Statistical analysis. A Student's t-test was done to demonstrate that there was no significant difference in baseline vocalizations between NOSD and SD mice. A repeated-measures (trial) ANOVA with one between factor (defeat or no defeat) was used to determine whether there were any significant differences in ultrasonic vocalizations to female cage bedding. As in Experiment 1, this analysis included power analysis and Mauchley's test of sphericity. If sphericity was rejected, the degrees of freedom were adjusted using Huynh–Feldt epsilon. Data were further probed using the least significance difference t-test. For territorial urine marking, a Student's t-test was performed between SD and NOSD mice.

Results

Two mice from each group failed to vocalize to a female during social experience tests, and were omitted from the study. There was no significant difference between NOSD and SD mice in the number of baseline ultrasonic vocalizations, $t(16) = 0.46$, $p = 0.66$. Mice that received repeated social defeats displayed fewer vocalizations than did nondefeated mice, $F(1, 16) = 5.59$, $p = 0.03$; Power $= 0.60$; see Fig. 2). There was no significant effect of test day, $F(3, 36) = 1.92$, $p = 0.13$; Power $= 0.50$. There was a significant interaction between defeat status and test day, $F(3, 36) = 2.96, p < 0.04$; Power = 0.73. Pair-wise comparisons revealed that ultrasonic vocalizations were significantly lower in SD mice relative to NOSD mice 30 min post-SD1 ($p < 0.05$) and 30 min post-SD2 ($p < 0.02$). There was a trend for fewer vocalizations in SD mice relative to NOSD mice 30 min post-SD3 ($p < 0.08$), but not 24 h post-SD3 ($p < 0.40$) or 72 h post-SD3 ($p < 0.40$). In addition, mice that received 3 repeated days of SD displayed less territorial urine marking 4 weeks after the third day of SD, $t(20) = 2.80, p < 0.02$ (see Fig. 3).

GENERAL DISCUSSION

There is a striking relationship between social dominance and the occurrence of male-typical androgen-dependent behaviors in house mice. For example, dominant males are more aggressive than subordinates, establish and defend territories (13), and sire more offspring (15). We currently report a prolonged decrease in territorial urine marking and a transient decrease in ultrasonic courtship vocalizations following SD. Mice that received acute SD displayed significantly less territorial urine marking than NOSD mice. In Experiment 2, SD mice displayed less territorial urine marking than did NOSD mice when tested 4 weeks following the last of 3 successive days of SD. These findings are not surprising because the frequency and pattern of territorial urine marking are strongly dependent on dominance status, with dominant mice marking more than subordinate mice (16). However, the prolonged effects of SD on territorial marking are surprising. Matthews (38) reported that individually housed, subordinate albino male mice displayed decreased territorial urine marking compared to dominant male mice when tested 6 days, but not 42 days, after the dominance relationship was established. In Experiment 1, we tested mice once per week in attempt to observe the time course necessary for territorial urine marking to return to normal levels following SD. We had hypothesized that over time, territorial marking in SD mice would reach that of NOSD mice, and that a second defeat would once again inhibit territorial marking in SD mice. However, territorial urine marking increased with repeated tests in both NOSD and SD mice, and marking levels in SD mice did not reach the level of NOSD mice. Although the second defeat experience at Week 6 tended to further decrease territorial marking, the level of marking in SD mice was still significantly lower than that of NOSD mice 5 weeks post-SD, prior to the second defeat experience (see Fig. 1).

The increased territorial marking with repeated tests is in agreement with findings by Matthews (37,46). Bronson and Desjardins (9) observed decreased marking with repeated tests, and suggested that as the novelty of the test and environment wear off, mice habituate and mark less, regardless of the presence of other animals. The different findings could be related to differences in methodologies, such as housing conditions. In Experiment 1 and in Matthews' study (37), mice were housed individually rather than pair housed as in the study by Bronson and Desjardins (9). Because it is well established

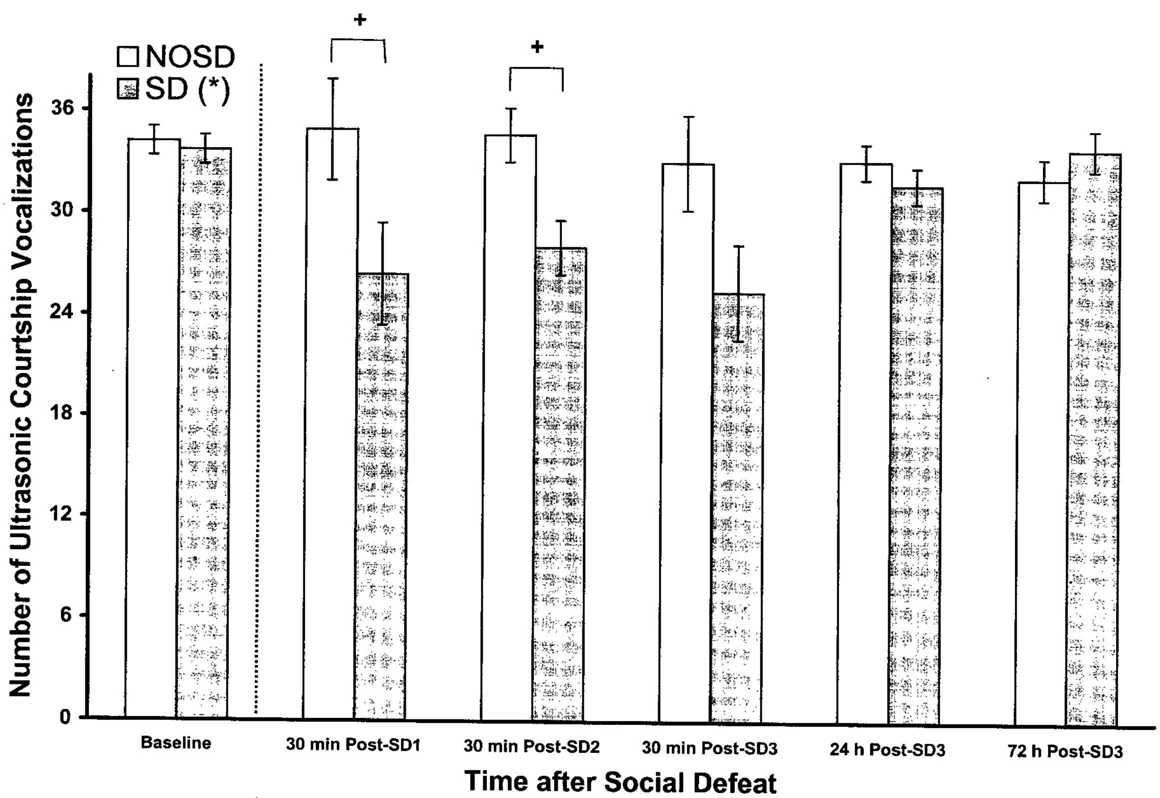

FIG. 2. Experiment 2: There was no significant difference in predefeat (baseline) ultrasonic courtship vocalizations. DBA/2 male mice that received 3 successive days of social defeat (SD; $n = 9$) displayed fewer ultrasonic courtship vocalizations than nondefeated mice (NOSD; $n = 9$) ($^*p = 0.03$). There was an interaction between defeat status and test trial ($p < 0.04$). At 30 min post-SD1 and 30 min post-SD2, SD mice displayed significantly fewer vocalizations than NOSD mice (significant differences are shown in brackets; $+p < 0.05$).

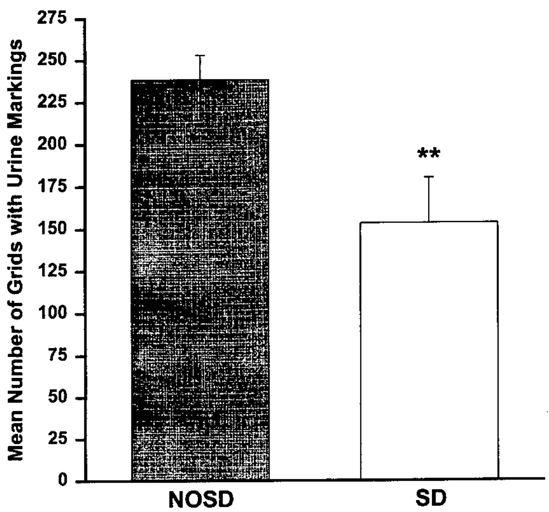

FIG. 3. Experiment 2: DBA/2 male mice that received 3 successive days of social defeat (SD; $n = 11$) displayed less territorial urine marking 4 weeks post-SD than did nondefeated mice (NOSD; $n = 11$) (**$p < 0.02$).

that isolation promotes aggression [reviewed in (7)], as well as neurochemical and hormonal changes, the increased marking seen during repeated tests might be secondary to individual housing of subjects. Strain differences could also account for the differences in findings, because differences in fear-motivated behaviors in inbred mouse strains have been reported (62). In the present study, DBA/2 mice may have displayed increased marking as their level of anxiety to being placed in an unfamiliar environment decreased.

Reduced territorial marking may be considered maladaptive in that the mouse does not claim a territory and readily pass on its genes. However, reduced marking may be adaptive to the individual in that it may decrease detection and attack, and thereby promote individual survival. Control in a social situation has been defined as "the successful avoidance of attack by a dominant male" (29). Reduced marking may be a coping strategy that is adaptive for the individual.

In many species, including rats (2,8,17), hamsters (25), monkeys (54,56), and humans (12,18), dominant males have higher testosterone levels. Although the relationship between testosterone levels and dominance is not as clear in mice (3,19,24,58), dominant male mice have been reported to have higher androgen levels than subordinates (35,36,39). In support of this finding, we observed that long-term individually housed C57BL/6 aggressor mice had higher testosterone levels than long-term individually housed nonaggressor mice (unpublished data). In addition, testosterone implants augmented aggression in mice, relative to vehicle-implanted mice (58). Although Bronson and Desjardins (10) reported that repeated defeats suppressed androgen levels in subordinate mice, we have not observed an effect of acute or repeated SD on testosterone levels (unpublished data), and therefore, did not measure testosterone levels in the mice tested in the current experiments. High individual variability in androgen levels in mice (4,35), strain differences in androgen levels, and the single time point collection of plasma in most mouse studies may contribute to the inconsistencies observed in testosterone levels in mice. Other physiological consequences of SD, including activation of the autonomic nervous system, alteration in circadian rhythms, and stress-induced release of neurohormones including corticosterone, β-endorphin, and norepinephrine (6,25,30,31,33) might contribute to stress-induced inhibition of territorial marking, possibly independent of circulating levels of testosterone.

A potential mediator of the decreased territorial marking following SD is the effect of severe stress on bladder distension. Desjardins et al. (16) reported that subordinate mice had more urine in their bladders. We have also observed increased bladder volume 24 h following SD in DBA/2 mice (unpublished data). Henry, Meehan, and Stephens (23) reported that chronic stress in mice leads to nephropathy, and hypothesized that renal failure may be associated with repeated episodes of urinary reflux. In hamsters, social stress leads to increased c-*fos* in the medial preoptic area (MPOA) (28). Restraint stress in rats increased c-*fos* in the same brain regions as administration (i.c.v.) of CRH, including Barrington's nucleus (26). The MPOA projects to Barrington's nucleus (53,57), which regulates urinary bladder control (32). MPOA projections to Barrington's nucleus might inhibit micturition during reproduction (59). Therefore, neural circuitry that regulates male sexual behavior feeds directly into circuitry that regulates bladder function. The potential relationship between stress-induced activation of Barrington's nucleus and prolonged impairment of territorial marking is unclear.

In Experiment 2, mice that received SD displayed fewer vocalizations to female cage bedding than did NOSD mice. The decrease in vocalization following SD was transient, as demonstrated by the significant interaction between the defeat and test trial. SD mice had significantly fewer vocalizations than NOSD mice at 30-min post-SD1 and 30-min post-SD2, and tended to have fewer vocalizations post-SD3. Vocalizations were elevated to that of NOSD mice at 24-h post-SD3. The finding that SD decreased ultrasonic vocalizations is not surprising, because dominant males have been shown to vocalize more than subordinate males (47). However, it is surprising that the effect of SD on ultrasonic vocalizations was short-lived. Given the prolonged changes in territorial urine marking following SD, we predicted a more dramatic effect on ultrasonic vocalizations.

Different mechanisms may regulate courtship vocalizations and territorial marking (49,55). The different time course of SD effects on these two behaviors further supports this suggestion. Castration decreases the frequency of both behaviors, and testosterone replacement restores these behaviors to precastration level (60). However, intracranial implants of testosterone into the MPOA, an important regulatory site for male sexual behavior [reviewed in (40)], are sufficient to restore courtship vocalizations completely, but only partly restore territorial marking (60). MPOA lesions do not affect courtship vocalizations in mice (5). Although we were unable to find literature on the effects of MPOA lesions on territorial urine marking, MPOA lesions in Syrian hamsters were reported to inhibit scent marking (1). Although the MPOA is not necessary for courtship vocalizations (5), androgens implanted into the MPOA are sufficient to reinstate courtship vocalizations in a castrated mouse (60). Clearly, courtship vocalizations are more resilient than territorial urine marking, to a variety of experimental manipulations, including social defeat. Further examination of SD-induced inhibition of vocalizations and territorial marking may be useful in elucidating the underlying neural circuitry and neurochemicals that regulate these behaviors, and may be a useful indicator for the development of pharmacological interventions of stress-induced maladaptive behaviors.

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30.6

BEHAVIORAL EFFECTS OF NAALADASE INHIBITION IN SOCIALLY DEFEATED MALE MICE.

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Social stress increases glutamate release in select brain regions. There has been recent interest in assessing anti-stress or anxiolytic effects of glutamatergic ligands. One source of glutamate is from the hydrolysis of N-acetylaspartylglutamate (NAAG) by the enzyme N-acetylated-α-linked acidic dipeptidase (NAALADase). The NAALADase inhibitor 2-(phosphonomethyl) pentanedioic acid (2-PMPA) inhibits conversion of NAAG to glutamate. We tested whether 2-PMPA (10 μg/day, i.c.v.), administered via osmotic mini-pumps 1 wk prior to social defeat (SD), would prevent defeat-induced behavioral changes in male mice tested in a resident-intruder test. Resident subject mice were tested for three 5 min. intervals (I): I1 (barrier), I2 (barrier plus non-aggressive intruder (NAI)), I3 (only NAI). Since there are strain differences in glutamate activity, we tested 2-PMPA in both DBA/2 and C57BL/6 mice. In Exp. 1, SD DBA/2 mice displayed more defensive posture, flight, avoidance, ear wiggling and risk assessment during I2 and/or I3, and fewer approaches, chase and attacks during I3 than non-defeated mice (NOSD) ($p<0.01$). 2-PMPA-treated mice had more approaches than vehicle-treated (VEH) mice during I2 ($p<0.05$). During I3, 2-PMPA increased stretch-attend posture ($p<0.01$) in SD mice and sniff NAI in NOSD mice ($p<0.05$). In Exp. 2, SD C57BL/6 mice displayed more defensive posture, flight, avoidance, tail raise and risk assessment during I2 and/or I3, and fewer approaches, chases and attacks during I3, than NOSD mice ($p<0.01$). C57BL/6 mice treated with 2-PMPA were more aggressive than VEH mice, as indicated by attack during SD or attack of the NAI during the resident-intruder test ($p<0.05$). 2-PMPA-treated mice groomed more than VEH mice during I2, and had more flat body approach (risk assessment) during I3 ($p<0.05$). In sum, 2-PMPA at this dose increased select indices of territoriality. Research was supported by DAMD-17-0041 between Guilford Pharm. Inc. and WRAIR.

30.8

PHARMACOLOGICAL EFFECTS OF PHENCYCLIDINE: DEPENDENCE ON NITRIC OXIDE AND Ca^{2+} SIGNALLING

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Phencyclidine (PCP) is a dissociative anaesthetic and psychotomimetic drug that may induce schizophrenia-like symptoms in humans which comprise both the positive and negative symptoms observed in schizophrenia. This unique property of PCP has rendered the "PCP model of schizophrenia" considerable scientific attention. Recent studies suggest that a nitric oxide (NO) dependent mechanism is implicated in the central effects of PCP. The NO synthase inhibitor, L-NAME, blocks several PCP-induced behavioural and biochemical effects in the rat. Since neuronal NO is generated from L-arginine by a Ca^{2+}-calmodulin-activated NO synthase, Ca^{2+} may be critically involved in the NO-dependent effects of PCP. In the present study the possible involvement of Ca^{2+}-induced Ca^{2+}-release (CICR) in the effects of PCP was investigated. CICR relies on the properties of the ryanodine receptor and represents a mechanism by which Ca^{2+} influx during neuronal activity can be amplified into larger intracellular Ca^{2+} signals. The ryanodine receptor agonist, caffeine, was shown to potentiate the locomotor stimulatory effect of PCP in a dose-

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THE ACOUSTIC STARTLE REFLEX, PRE-PULSE INHIBITION AND ALCOHOL CONSUMPTION IN RATS

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The relationship between DA and alcohol (EtOH) consumption is widely accepted, so is the association between pre-pulse inhibition (PPI) of the startle-reflex and the dopaminergic system. The aim of our project was to investigate if PPI, or other aspects of the startle-response, could predict later voluntary EtOH consumption, and whether animals with various degrees of EtOH preference would respond differently in the subsequent startle tests.

Fifty male Wistar rats served as experimental subjects. Three tests of the acoustic startle reflex were performed in a standard startle chamber (SR-LAB, San Diego Instruments). After an initial pre-test animals were assigned to one of two groups; high or low PPI. Half of the animals in each group were then offered the choice of EtOH (3, 5 and 7%) and water for 16 days, the other half received only water. After the EtOH regime was terminated, the animals were tested in a second (post-ethanol) test. Finally, after 4 weeks of EtOH abstinence, the animals were tested again (post-abstinence).

PPI failed to predict consumptive behavior. However, the amplitude of the startle-reflex after the *first* pulse alone (p-alone) (120dB) predicted later EtOH consumption. Rats with low amplitudes of the startle-reflex drank less than rats with a strong startle reflex. Habituation of the startle-reflex in the pre-test showed a relationship with EtOH, with animals showing the steepest habituation curves becoming the heaviest drinkers. Data revealed that high EtOH consumers showed lower PPI than low consumers/water controls in the post-ethanol test, a difference that was absent in the post-abstinence test. The effect of EtOH on the p-alone startle effect was more prolonged. All groups were showing similar amplitudes in the pre-test, and in the post-ethanol test this first p-alone response was increased with approx. 25 % for all groups. In the post-abstinence test, the water control and the low consumers showed a decreased, back-to-baseline response, while the heavy drinkers continued to increase their response further, to 37.5 % past baseline.

Obviously, there is a relationship between reactivity to one strong acoustic stimulus and EtOH drinking. Moreover, animals that react strongly, but show rapid habituation to the acoustic stimuli are more prone to ingest EtOH. EtOH has a short-term effect on PPI by reducing it, and affects the startle reflex in a more prolonged fashion by increasing it.

EFFECTS OF SOCIAL DEFEAT ON TERRITORIAL URINE MARKING IN C57BL/6 MICE IN RESPONSE TO MALE AND FEMALE STIMULUS MICE.

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Social stress induces marked physiological and behavioral changes, including effects on bladder function and on dominance status. Dominant mice extensively disperse territorial urine marking, whereas subordinate mice display minimal marking. In male DBA/2 mice, acute social defeat (SD) induces prolonged inhibitory effects on territorial urine marking in response to another male mouse. We presently report on effects of SD on territorial marking in male C57BL/6 mice in response to either a male stimulus mouse and/or a female stimulus mouse.

In Exp. 1, territorial marking was measured in SD and in non-defeated (NOSD) mice exposed to a male stimulus mouse and exposed to a female stimulus mouse. Pre-defeat territorial urine marking was higher to female mice than to male mice. Defeated male mice had decreased marking in the presence of a male stimulus mouse, but not in the presence of a female stimulus mouse ($p=0.01$). In Exp. 2, the time course of the effects of acute SD on territorial marking was examined by testing mice 1/wk for 5 wk in response to either a male or a female stimulus mouse, but not to both as in Exp. 1. After week 5, mice received a second SD and were tested 24 h later for territorial urine marking. They were then sacrificed, and tissues and plasma were collected. NOSD mice marked more than SD mice ($p<0.02$). Mice tended to mark more to female than to male mice, primarily since NOSD mice marked more to female than to male stimulus mice ($p<0.04$). NOSD mice exposed to a female had elevated corticosterone ($p<0.02$) and testosterone ($p<0.02$). SD mice had greater bladder volume than NOSD mice, regardless of gender of stimulus mouse ($p<0.03$), and a trend for greater adrenal weights. There was no difference in the body, testes or seminal vesicle weights. In sum, SD-induced changes in territorial marking also occur in C57BL/6 mice. Defeat-induced inhibition of territorial marking is initially impaired less in the presence of a female than a male mouse, and cannot be explained solely by stress-induced changes in bladder control. Lastly, exposure of NOSD male mice to females elicits hormone release.

PATHOLOGY REPORT

Walter Reed Army Institute of Research, Washington, DC

ACCESSION NUMBER 99-0742

ⓧJ1 was submitted Jul 13. 1999.

ACCESSION NUMBER	CONTRIBUTOR	DIVISION / DEPARTMENT			DATE OF REPORT
99-0742	LUMLEY	NEUROSCIENCE			13-JUL-99
PROTOCOL N05-99	**PROSECTOR** SLH	**DATE SUBMITTED** 7,ⓧ 07-JUL-99			**DATE COMPLETED**
SPECIES MOUSE	**STRAIN** C57B1/6	**AGE (mos)** 2.5	**SEX** M	**WEIGHT (kg)** .0296	**ANIMAL ID** J1

HISTORY

This animal was one of a large group of mice received in early June. Approximately 60 of 180 mice in this group are affected with abscesses in the preputial area. Staphylococcus aureus was cultured from abscess material. A few mice with abscesses have died. Animal was euthanatized by CO2 anesthesia followed by cervical dislocation.

MACROSCOPIC FINDINGS

Suabmitted for necropsy is a male mouse in good body condition as evidenced by normal stores of subcutaneous and cavitary body fat. A one cm diameter subcutaneous abscess filled with yellow purulent material that does not involve the body wall is present in the right inguinal area lateral to the opening of the prepuce. There is a 4 x 2 mm area of cutaneous ulceration over the abscess. The right subiliac lymph node is twice the size of the left and mottled tan and red. The stomach contains scant food material; small intestinal and cecal contents are unremarkable and the colon is filled with normal feces.

Abscess material and liver were submitted for bacterial culture.

MICROSCOPIC FINDINGS

Pending

COMMENTS

Gross lesions in this animal are limited to the inguinal abscess and associated local lymphadenopathy. The animal's normal body fat stores indicate recent adequate food intake. The lack of food in the stomach may indicate anorexia or lack of food. Staphylococcus aureus, which may be harbored on the skin, nasopharynx and intestine, is a relatively commonly reported cause of preputial gland abscesses in mice. Contributing factors include nutritional stress, barbering injury by cage mates, fighting injuries, skin mites and the environmental level of Staphyloccus. This is a preliminary report; final report will follow histopathology of selected tissues and bacterial culture.

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